July–August 1952 Number 4

The Journal of Infectious Diseases

Published for the John Rockefeller McCormick Memorial Institute for Infectious Diseases
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PUBLISHED FOR THE JOHN ROCKEFELLER McCORMICK MEMORIAL FUND
OF THE UNIVERSITY OF CHICAGO

THE UNIVERSITY OF CHICAGO PRESS
CHICAGO 37, ILLINOIS · U.S.A.

The Journal of Infectious Diseases

The Journal of Infectious Diseases is devoted to the publication of original investigations dealing with the general phenomena, causation and prevention of infectious diseases. It is the aim of the journal to restrict its material field and to include only such contributions as bear with reasonable directness on the topics indicated in the title. The biology and chemistry of the various pathogenic organisms, the physiology and anatomy of the morbid processes in the body follicle, and the hygienic and sanitary problems to which they give rise are considered to be especially within the scope of the undertaking.

This journal was established in connection with the John Rockefeller McCormick Memorial Institute for Infectious Diseases, by the munificence of the late Mrs. Edith Rockefeller McCormick and her son Mr. Harold F. McCormick. Since 1941 it has been published in connection with the John Rockefeller McCormick Memorial Fund of the University of Chicago.

The Journal of Infectious Diseases is published bi-monthly by The University of Chicago Press, 5750 Ellis Avenue, Chicago, Illinois. The subscription price is $6.00 per year; $3.00 a volume; the price of single copies of current volume is $1.00. ¶ Postage is prepaid by the publishers on all orders from the United States, Mexico, Cuba, Puerto Rico, Panama Canal Zone, Republic of Panama, Dominican Republic, Canary Islands, El Salvador, Argentina, Bolivia, Brazil, Colombia, Chile, Costa Rica, Ecuador, Guatemala, Honduras, Nicaragua, Peru, Haiti, Uruguay, Paraguay, Hawaiian Islands, Balearic Islands, Spain and Venezuela. ¶ Postage is charged extra as follows: for Canada and Newfoundland, 30 cents on annual subscriptions (total $6.30); on single copies 5 cents (total $1.05); for all other countries in the Postal Union, 60 cents on annual subscriptions (total $6.60); on single copies 10 cents (total $1.10). ¶ Patrons are requested to make remittances payable to the University Press in postal or express money orders or bank drafts.

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Claims for missing numbers should be made within the month following the regular month of publication. The publishers expect to supply missing numbers free only when issues have been sustained in transit and when the reserve stock will permit.

Business correspondence should be addressed to The University of Chicago Press, 5750 Ellis Avenue, Chicago 37, Illinois.

Communications for the editors and manuscripts should be addressed to Dr. W. H. Taliaferro, The University of Chicago, 5724 Ellis Ave., Chicago 37, Ill.

Entered as second-class matter August 1, 1911, at the post-office at Chicago, Illinois, under the Act of March 3, 1879.

Acceptance for mailing at special rate of postage provided for in the United States Postal Act of October 3, 1917, Section 1103, amended February 28, 1925, authorized August 7, 1942.

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J. INFECT. DIS. VOL. 91, NO. 1

SALMONELLOSIS IN DOGS

I. BACTERIOLOGICAL, EPIDEMIOLOGICAL AND CLINICAL CONSIDERATIONS

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Most salmonella infections are primary infections of animals or birds which may be transmitted to man. There are numerous reports in the literature referring to the epidemiological significance of infection in domestic fowl, swine and wild rodents; in contrast, there have been relatively few studies of the occurrence of these infections in domestic pets which live in such intimate association with man.

BACTERIOLOGICAL PROCEDURES

All fecal specimens were obtained by rectal swab, collected by a bacteriologist, physician or veterinarian associated with the study and were planted directly into an enrichment broth. Tetrathionate broth was used throughout, but the method of preparation varied from time to time as described below. The inoculated broths were incubated for 18 to 24 hours at 37 C and then streaked to brilliant green agar plates. Early in this study, SS agar was used also for comparative purposes. Ordinarily, from two to four suspicious colonies per specimen were picked. If a plate appeared suspicious but no isolated colonies were present, the enrichment was restreaked to a fresh plate using a smaller inoculum, or the suspicious growth was transferred directly to a fresh plate and streaked for isolation. Those KIA* slants which showed a reaction characteristic of one of the salmonella group (alkaline slant, acid and gas in the butt with or without hydrogen sulfide) were first screened serologically with a polyvalent salmonella serum† in a slide test. If a positive reaction was obtained, the culture was typed serologically according to the Kauffmann-White schema. If the culture was negative in the slide agglutination test, it was inoculated into urease medium and tryptone broth. All urease and indol-positive cultures were discarded. Those negative in both tests and hydrogen sulfide-positive were further screened by slide agglutination with a polyvalent *Paracolobactrum intermedium* serum and a pooled Arizona paracolon serum. Isolations of questionable nature and representatives of all unusual types were sent to Dr. P. R. Edwards, Communicable Disease Center, Chamblee, Georgia for further examination.

The KIA cultures which showed an alkaline slant, acid butt and no gas, with or without hydrogen sulfide, were screened biochemically and serologically according to the procedure outlined by Galton et al.[1] Those cultures that appeared to be anaerogenic salmonella were typed as described above.

Biochemical screening of KIA slants characteristic of salmonella was found inadvisable. The urease test was performed on all of the suspicious KIA slants subsequent to "spotting" with polyvalent salmonella serum. In a series of 322 specimens which were found positive for salmonella,

Received for publication October 24, 1951.

These studies were supported through a grant from the Armed Forces Epidemiological Board.

Appreciation is due the kennel owners, the Florida Racing Commission, the practicing veterinarians, the Bureau of Vital Statistics, Mr. Everett H. Williams, Director, and to Mr. L. E. McEldowney, Chemist, Bureau of Laboratories, for their cooperation during this work. Antibiotics used in therapeutic trials were supplied by Lederle Laboratories, Parke, Davis & Co., and Chas. Pfizer & Co., Inc.

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* Kligler's iron agar with 1% sucrose added.

† All salmonella and paracolon serums, with the exception of *P. intermedium* serum, were provided by Dr. Edwards.

1. Galton, M. M., Hardy, A. V. and Mitchell, R. B. 1950, The public health laboratory diagnosis of enteric infections. Am. J. Trop. Med. **30**: 77–90.

52 (16.1%) contained proteus as a contaminant. Had urea medium been employed as an initial screening test in this series, these would have been discarded. The use of mannite for screening also was unsatisfactory for the same reason.

Comparative studies to evaluate the relative efficacy of procedures have been conducted during the study. Two swabs were taken simultaneously and these were cultured by differing techniques. There have been the following findings. Tetrathionate prepared according to the Kauffmann formula proved a little less effective than that prepared from Difco dehydrated reagent with the addition of brilliant green (1 ml of 1:1000 solution per 100 ml medium). Sulfathiazole, 0.125 mg per 100 ml, was added to each of these preparations to prevent excessive overgrowth of the proteus. Of the 608 specimens examined comparatively, 154 (25.3%) yielded salmonella from the Kauffmann tetrathionate and 176 (29.0%) from the Difco reagent with brilliant green.

The manufacturer of the dehydrated enrichment recommends that the iodine solution be added the day the broth is used. It is much simpler to add this in bulk before the medium is tubed. Two preparations of tetrathionate medium were prepared and used in parallel, one with the iodine added on the day of use and one with it added during its preparation, 3 to 14 days earlier. With the former there were 73 positives and with the latter 72 positives. Though there was no difference here, there appeared to be a substantial decrease in efficacy when the iodine was added from 17 to 25 days before use. In a smaller series of 59 specimens, 12 were positive using the old preparation while 22 were found on using a fresh tetrathionate with iodine added on the day of its use.

The most troublesome problem in processing fecal cultures of dogs is the overgrowth by proteus. The addition of sodium sulfathiazole solution in a low concentration (0.125 mg per 100 ml) appeared to have some value in retarding the growth of proteus in the enrichment without inhibiting salmonella. When this was employed in Kauffmann's tetrathionate, the sulfonamide preparation proved superior to the unmodified enrichment, but using Difco tetrathionate in a series with 198 positive specimens, there were 181 positives from the sulfonamide modification as compared with 176 for the unmodified enrichment. Attention has been given also to the possible value of tibatin,[2,3] sulfamylon* and sulfadiazine in preventing overgrowth with the commonly occurring pseudomonas organisms. Further studies are needed to adequately evaluate these and comparable modifications in technique.

In these studies brilliant green agar has been superior to SS as a medium for plating from the enrichment. In a series of 286 specimens, 72 specimens in all were found positive. There were 71 positives from the brilliant green agar and 27 from SS, and in only one culture were the organisms isolated from SS only.

The value of enrichment mediums in the isolation of salmonella is accepted, but in 255 specimens, plates were inoculated directly to brilliant green agar for comparative purposes. By enrichment there were 230 positives and by direct plating only 105.

TABLE 1.—*Cultural findings for salmonellosis in dogs in Florida.*

Animals examined	Specimens cultured	Number positive	Percent positive	Salmonella isolations	
				Number	Per 100 cultures
Normal dogs	1626	244	15.0	257	15.8
Veterinary hospitals, pounds and boarding kennels	3459	618	17.9	701	20.3
Greyhounds—Survey cultures	2548	930	36.5	1129	44.3
Treatment series	524	460	87.78	612	116.79
Total	8157	2252	27.6	2699	33.1

GENERAL OBSERVATIONS

In all there were 8157 cultural examinations on dogs, of which 2252 (27.6%) were positive for salmonella, as summarized in table 1.

However, the variability in findings was the striking feature. The proportion

2. Altenbach, G. 1949, Hemmung von Bakterium Coli und Proteus durch Tibatin auf festen Nährboden ohne Schädigung anderer Bakterien. Zentralbl. f. Bakt. **154**: 135–139.
3. Henkel, G. 1949, Über den Einfluss von Tibatin auf das Wachstum von Bacterium coli und Streptokokken in flüssigen Nährboden. Zentralbl. f. Bakt. **154**: 140–145.

* Provided by the Sterling-Winthrop Research Institute, Rensselaer, N. Y.

of positives in different groups or communities ranged from very low to very high, and this was true even in the same hospital or kennel in which all animals were examined repeatedly. The continuously confined greyhounds had very high infection rates. There were multiple isolations of salmonella from 412 specimens. Of these multiple infections, 326 occurred in greyhounds, 73 in hospitalized and impounded dogs and 13 in normal dogs. There were 379 double infections, 31 triple and two quadruple. The isolation of two types from a single colony was not unusual.

Evidence was repeatedly encountered indicating that salmonella infections of dogs are of relatively short duration. It was apparent that dogs must be exposed to salmonellosis very frequently. The data for greyhounds suggested an average of more than one infection per month. Likewise, the wide variation in types of salmonella encountered pointed to a constantly changing type of infection, with high rates maintained by frequent exposures.

There was some clear evidence as to modes of dissemination. Salmonella were isolated from foods ready for feeding in two veterinary hospitals. The types of salmonella corresponded with those prevalent in the hospital at the time. Furthermore, the rapid and apparently simultaneous spread in other hospitals or kennels indicated some common vehicle with a heavy dose of salmonella. There was much evidence pointing toward food-borne infections with salmonella, in the two instances clearly confirmatory findings. However, mass dissemination through common contaminated foods could account for a portion of the infections only. There was virtually no evidence to suggest the sources and modes of infections of the family dog.

The types of salmonella isolated are considered here for their epidemiological significance only. During these studies 52 serological types were found, including three new types, seven not previously encountered in Florida and one monophasic variant. The type distribution of those isolated from dogs as compared with those from man in Florida are given in table 2. There is a notable similarity in distribution. Variations are explained in part by localized outbreaks, as for example, many of the isolations of *S. miami* from humans occurred during the epidemic in the city whose name it bears. The data strongly suggest that salmonella infections in humans and dogs in Florida in part are either derived from common sources, are spread from animal to man, or possibly the reverse.

Certain findings of bacteriological interest were noted. Of the 20 cultures of *S. paratyphi B*, ten were diphasic, ten were monophasic and all were D-tartrate negative. Both are frequently associated with enteric fever in man. Eight cultures of *S. minnesota* were monophasic, as were two of *S. urbana*. There were six isolations of anaerogenic strains of *S. muenchen* from five dogs and one cat. On original isolation these produced a small amount of hydrogen sulfide only and simulated *S. typhosa*. These cultures were obtained from animals at three hospitals, one greyhound kennel and a rabies clinic over an 18-month period. The occurrence of 12 hydrogen sulfide-negative strains of *S. canoga* was noted.

Paracolons of the Arizona group were encountered in five pound dogs, four in veterinary hospitals and two greyhounds. The same antigenic type, Ar. 10:1,2,5, was obtained from ten of these animals; Ar. 9:1,3,11 was isolated once from a hospitalized dog. Members of the *Paracolobactrum intermedium* (Bethesda) group were encountered frequently in all groups of dogs.

TABLE 2.—*Distribution of salmonella types in dogs and man in Florida.*

Group	Type	Dogs				Man
		Greyhounds	Impounded, hospitals and boarding kennels	Normal	Total	
B	1. *S. paratyphi B*	6	9	5	20	30
	2. *S. saint paul*			15	15	16
	3. *S. san diego*			5	5	19
	4. *S. derby*	199	35	4	238	96
	5. *S. california*	5	2		7	
	6. *S. typhimurium*	20	54	6	80	141
	7. *S. bredeney*	25	34	16	75	30
C_1	8. *S. cholerae suis*	1	3		4	22
	9. *S. norwich*			1	1	2
	10. *S. montevideo*	22	56	4	82	94
	11. *S. thompson*		2		2	4
	12. *S. daytona*					2
	13. *S. oranienburg*	60	34	7	101	122
	14. *S. bareilly*	8	9	6	22	20
	15. *S. hartford*	2	2	7	11	20
	16. *S. VI VII y—*					2
	17. *S. tennessee*	10	27	2	39	24
C_2	18. *S. gatuni*					1
	19. *S. quiniela*	1			1	
	20. *S. muenchen*	15	14	13	42	28
	21. *S. manhattan*	2	64	2	69	15
	22. *S. newport*	50	28	10	86	112
	23. *S. bonariensis*			1	1	3
	24. *S. litchfield*	2	10	8	20	29
	25. *S. bovis-morbificans*					3
	26. *S. tallahassee*		1	18	19	8
	27. *S. kentucky*	1	8	2	11	
D	28. *S. miami*	3	14	20	37	80
	29. *S. eastbourne*					1
	30. *S. berta*					17
	31. *S. enteritidis*		1		1	1
	32. *S. pensacola*					2
	33. *S. panama*					26
	34. *S. javiana*	1	7	5	13	5
	35. *S. pullorum*					2
E_1	36. *S. butanan*					1
	37. *S. anatum*	467	62	21	556	145
	38. *S. nyborg*	1			1	
	39. *S. meleagridis*	2			2	29
	40. *S. give*	30	18	2	50	24
E_2	41. *S. newington*	8	4	1	13	23
	42. *S. newbrunswick*			1	1	4
	43. *S. canoga*	13	3	1	17	
	44. *S. illinois*	24	4		8	
E_3	45. *S. senftenberg*	27	59	4	90	12
F	46. *S. luciana*		1	3	4	4
	47. *S. aberdeen*					2
	48. *S. rubislaw*	70	16	15	101	11
G	49. *S. mississippi*					1
	50. *S. XIII XXII XXVI b—*		1		1	
	51. *S. worthington*	6	41	3	50	2
	52. *S. poona*	4	12	3	19	10
	53. *S. cubana*		25	4	29	1
H	54. *S. florida*			10	10	5
	55. *S. carrau*	2	1	8	11	6
	56. *S. madelia*	1	15	3	19	7
	57. *S. homosassa*	1		6	7	1
I	58. *S. gaminara*		4	5	9	
	59. *S. memphis*		1		1	
FG	60. *S. cerro*	19	6	3	28	6
	61. *S. minnesota*	17	3	3	23	24
	62. *S. pomona*			3	3	
	63. *S. urbana*	4	2		6	5
	64. *S. inverness*			1	1	1
	65. *S. duval*		3		3	
	66. *S. allandale*					1
Total isolations		1129	701	257	2087	1303
Number of types		36	41	41	53	56

In limited studies of the human contacts of known infected animals, no evidence was obtained to indicate that dogs are a common source of human infections. This possibility needs to be examined further.

There also was no conclusive evidence as to the significance of salmonella infections to the dogs themselves. Most of the animals when found infected were healthy. Some were acutely ill with what is commonly termed "kennel sickness." This entity, generally recognized as being particularly troublesome in the young, is characterized by an early fever, a diarrhea of varying severity and not infrequently accompanying signs of respiratory infection. We have been impressed with the similarity of this disease entity and clinical salmonellosis in children. It has been our opinion that experimental studies to demonstrate the clinical significance of salmonellosis in dogs and its possible relation to the common and serious infectious disease known as "kennel sickness" are clearly warranted.

Detailed observations are presented in the three following papers in this series. Though greyhounds are an unusual group of dogs, they are closely controlled and could be studied readily. Certain observations could be obtained there which were unobtainable in the constantly changing populations of animal hospitals, pounds and boarding kennels, or in dogs in their usual home environment. In these latter groups it was rarely practicable to examine the same animal repeatedly. The data on greyhounds are given in the next paper, followed by that on dogs in animal hospitals, pounds and boarding kennels. The last paper in the series reports findings on the normal family dog.

SUMMARY

1. Procedures used in the isolation of salmonella from dogs are described.

2. The relative efficacy of various laboratory procedures were compared.

3. Proteus organisms were observed as a prevalent contaminant in canine feces.

4. Of 8157 rectal swabs from dogs, 2252 (27.6%) were positive for salmonella.

5. The proportion positive in different groups or communities ranged from very low to very high.

6. A total of 53 serologic types, including three new types, were encountered. Multiple types were found in 412 specimens.

7. Salmonella types which were isolated frequently from dogs were also encountered frequently in man.

8. Evidence did not permit conclusions as to the significance of salmonella infections in dogs either as a source of spread to man or as a cause of disease in dogs.

SALMONELLOSIS IN DOGS

II. PREVALENCE AND DISTRIBUTION IN GREYHOUNDS IN FLORIDA

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Greyhound racing is an industry as well as a recreation in Florida. Tracks are located in the major population centers, with four in the Miami area and two in Jacksonville. Several hundred greyhounds are in the state for a part or all of the year. The observations reported here were made chiefly in Jacksonville, where the Central State Public Health Laboratory is located. For comparative purposes some cultures were obtained in Miami.

Greyhounds are well disciplined and carefully controlled dogs. Most of their lives are spent in kennel units. From the time of their birth they are handled, trained and managed on a fixed schedule. Feeding, exercise, sleep and even the passing of stool and urine occur at regular hours of the day and night. Except when the greyhound is ill, no excrement is passed in the kennel itself.

In general, the dogs of each owner or trainer are a well isolated group, except when participating in racing schedules. The animals are then taken to the tracks, housed in individual kennel units in a common building, and are brought together only during the preparation for and the running of the race. These operations are supervised by an attending veterinarian, who is responsible for the sanitary management.

Received for publication October 24, 1951.

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Fecal cultures from greyhounds in Jacksonville were taken in the manner described and examined by the technique specified in the first paper of this series. It was the usual practice in the survey examinations to culture all animals in the kennel, though in a few instances on request, a portion only were examined. Repeat surveys, when obtained, were taken at approximately monthly intervals. The samples from Miami were taken only during the period from March 17 to April 17, 1950. There each kennel was visited only once.

Salmonella were isolated from greyhounds in 14 of the 15 Jacksonville kennels examined. Only 18 dogs were cultured in the one cultural survey in the kennel with completely negative findings. On the first sampling in another kennel all 20 dogs tested were negative. Three weeks later five types of salmonella were found in a total of ten isolations, and at a later time all samples taken were positive. In all, 1602 survey cultures were taken from Jacksonville kennels, of which 697 (43.6%) were positive for salmonella. The variations in findings by kennels are given in table 1. There were additional wide fluctuations on the repeat examinations in the same kennel. In kennel *H*, for example, there were no cultural positives in one survey in which 31 dogs were tested, and in two other surveys less than 10% were positive. At the other extreme, on one survey in the same kennel all 45 animals tested were positive. The remaining four tests in this kennel re-

TABLE 1.—*Findings by survey cultures for salmonellosis in 15 greyhound kennels in Jacksonville.*

Kennels	Examinations	Positive specimens		Isolations			
		Number	Percent	Number	Per 100 specimens	Types isolated	Times sampled
A	43	29	67.4	35	81.4	6	2
B	263	153	58.2	215	81.7	17	10
C	14	8	57.1	9	64.3	5	1
D	7	4	57.1	4	57.1	2	1
E	61	32	53.0	37	60.7	6	3
F	171	86	53.0	108	63.2	15	8
G	66	31	47.0	35	53.0	4	4
H	295	136	46.1	184	62.4	11	8
I	75	31	41.3	40	53.3	8	2
J	48	17	35.4	18	37.5	5	2
K	27	9	33.3	9	33.3	4	2
L	387	122	31.5	137	35.4	21	11
M	104	32	30.8	39	37.5	6	5
N	23	7	30.4	8	34.8	3	3
O	18	0	0	0	0	0	1
Totals	1602	697	43.5	878	48.6	35	

vealed prevalance rates in the approximate range of 40 to 80%. Findings of like nature, though less extreme, were observed in all kennels.

The 946 Miami samples were taken from 43 different kennels. There were less than 10 dogs in 2 kennels, 10 to 19 in 23, 20 and 29 in 10, 30 to 39 in 5, and one each had 41, 54 and 82 dogs. There were no positives in each of 7 kennels in which a total of 124 cultures were taken. Over 80% were found positive in 2 kennels in which 37 cultures were taken. The proportion of positives in the remainder were 5 to 19% in 14 kennels, 20 to 39% in 6, and 40 to 59% in 14 kennels. Considering all specimens taken in Miami, 233 (24.6%) were positive for salmonella. During the same months in Jacksonville the proportion positive was 22.6%, a rate lower than the average for that area.

Through the generous cooperation of certain kennel managers in Jacksonville, it was possible to get repeat examinations on many animals. The findings according to the number of examinations per animal are given in table 2. The proportion of positive animals increases with repeated observations. Of the 98 which were cultured five or more times, 96 were found positive for salmonella. However, the proportion of the specimens tested which were found positive fluctuated within narrow limits only. A total of 27 dogs were cultured nine or more times each, but 23 of these were in one very well-managed kennel where the owner was particularly interested in the study and very coopera-

TABLE 2.—*Findings by survey cultures for salmonellosis by number of examinations per animal in greyhound kennels in Jacksonville.*

Examinations per dog	Dogs examined	Dogs positive		Specimens examined	Specimens positive		Total isolations	
		Number	Percent		Number	Percent	Number	Number per 100 examinations
1	250	117	46.8	250	117	46.8	139	55.6
2	133	94	70.7	266	116	43.6	143	53.8
3	52	43	82.7	156	69	44.2	88	56.4
4	39	37	94.9	156	60	38.5	80	51.3
5	23	23	100.0	115	55	47.8	73	63.5
6	6	6	100.0	36	16	44.4	22	61.1
7	13	13	100.0	91	41	45.1	52	57.1
8	29	28	96.6	232	121	52.2	163	70.7
9 and over	27	26	96.3	300	102	34.0	118	39.3
Totals	572	387	67.7	1602	697	43.5	878	54.9

TABLE 3.—*The incidence of salmonellosis among greyhounds in Jacksonville.*

Months of observation	Dogs examined	Positive		Observation period ("dog years")	Total infections and reinfections observed	Annual attack rate per 100 dogs
		Number	Percent			
1	267	131	49.1	22.2	158	712
2	135	98	72.6	22.4	141	629
3	61	51	83.6	15.3	104	680
4	23	22	95.7	7.7	36	468
5	19	19	100.0	7.9	52	658
6	11	11	100.0	5.5	40	727
7	20	20	100.0	11.7	87	744
8	18	18	100.0	12.0	76	633
9 and over	18	17	94.4	15.2	59	388
Total's	572	387	67.7	119.9	753	628

tive. The percentage of positive specimens here was lower than the average. Multiple types of salmonella were isolated from some specimens; hence the isolations per 100 specimens as given in the last columns of table 2 are higher than the percent of specimens found positive.

There are limited data which may be used to estimate minimum attack rates. Survey cultures were taken at approximately monthly intervals. Findings were tabulated according to the number of different months in which there was at least one cultural observation. Again it is seen in table 3 that the longer the dog was observed, the higher the proportion found positive. For purposes of computing incidence rates, if a dog was cultured during a month, the findings were accepted as indicating the occurrence or nonoccurrence of salmonellosis during that month. Thus, if a dog is cultured in eight months, that animal is considered to have been observed for eight months. The total period of observation and the total infections acquired for all dogs were computed in this way. The total period of observation in terms of observation years is given in table 3. The total number of salmonella infections plus apparent reinfections are also shown. If an animal had been free of an infection for more than three months with bacteriological negative cultures intervening, a later reappearance of that type was counted as a reinfection. There were 712 infections and 41 reinfections, giving the total of 753 infections and reinfections. The average annual attack rate is 628 salmonella infections per 100 dogs per year or about one infection each two months. The computed rates by months of observation are strikingly constant except for the lower rate in the group observed over eight months, again almost entirely from the one superior kennel.

TABLE 4.—*Findings for salmonella on 44 dogs cultured repeatedly in one Jacksonville greyhound kennel.*

Date of Culture	*Salmonella* isolated					Total
	anatum	*derby*	*newington*	*poona*	*montevideo*	
9–25–50	44	25	0	3	0	72
9–29–50	36	28	0	2	0	66
9–30–50	36	15	0	0	0	51
10–1–50	33	29	0	0	0	62
10–2–50	9	5	33	1	0	48
10–3–50	31	21	3	0	0	55
10–4–50	43	12	5	0	0	60
10–5–50	32	15	1	1	0	49
10–6–50	36	17	0	0	0	53
10–9–50	38	13	0	14	0	65
10–12–50	24	5	1	0	0	30
10–16–50	12	3	3	1	0	19
10–23–50	41	10	0	0	1	52
Totals	415	198	46	22	1	682

Data in table 4 from one kennel indicate the possible relationship of rates computed on the basis of cultural observations once monthly and those obtained by repeated cultures. Salmonella infection was widely prevalent among the 44 dogs in this kennel. Special attention was given to this group since on September 25th, all animals were positive for *S. anatum*, 25 also for *S. derby*

and 3 for *S. poona*. Within the month 12 additional repeat cultures were taken. (These special examinations are not included as a part of the survey cultures.) The total number of infections detected by one cultural examination varied from the 72 on September 25th to the low of 19 on October 16th, and the average for the 13 cultural surveys was 52. In the period of observation and by the tests used, 44 dogs were found infected with *S. anatum*, 43 with *S. derby*, 37 with *S. newington*, 18 with *S. poona*, and 1 with *S. montevideo*, a total of 143 known infections in the 44 animals in the month, a rate of 3.2 infections per dog per month, or 3840 salmonella infections per 100 dogs per annum. Thus, the minimum incidence measured by cultures once monthly is much less than the true incidence. The degree of difference would be even greater than that indicated had the infections been less prevalent. There was a total of 22 isolations of *S. poona* in the 13 examinations, an average of 1.7 per test. However, in the month 18 different dogs were found infected, an incidence more than ten times that indicated by an average single survey. Thus it can be assumed that the true attack rates for salmonellosis in the greyhounds observed were substantially higher than one attack every two months. The data suggest rather that the true attack rates may be in the order of three infections per greyhound per month.

There was a moderate variation in prevalence by season. Cultures were taken only when the kennel owner gave his permission and very few were interested in repeated tests. However, a majority of the specimens were taken from five kennels which were examined from 5 to 11 times each. The proportion of the Jacksonville cultures found positive by month is shown in table 5. Racing at the local tracks began in late

TABLE 5.—*The seasonal prevalence of salmonellosis in Jacksonville, October, 1949 through November, 1950.*

Date	Cultures	Positive	
		Number	Percent
1949			
October	89	45	51
November	345	178	52
December	177	99	56
1950			
January	144	88	61
February	120	38	32
March	115	25	22
April	93	22	24
May	86	16	19
June	78	18	23
July	122	38	31
August	69	18	26
September	77	61	79
October	39	34	87
November	38	16	42
Totals	1512	696	46

November and continued through June. A high rate of infection was found in the late fall and early winter, with much lower rates in the late winter, spring and summer. It is noted further that the rates became elevated before the racing season began and fell to a low rate while racing was still in progress. The findings do not support the commonly expressed opinion that the infections must be spread through animal contacts at the tracks.

To examine the possible correlation of the prevalence of infection with cleanliness and sanitation in the individual kennels, each kennel was graded as good, fair or poor. This was done independently by two bacteriologists, each of whom had visited the kennels repeatedly. Their classifications had only minor differences. The cleaner kennels had lower rates. However, even in the clean kennels 32% of the specimens taken were positive. In kennels with cleanliness graded as fair, 42% were positive, while in those rated as poor the prevalence rate was 55%. Poor sanitation may have an influence on the spread of the infection, but other factors may be of even greater importance.

Through the cooperation of biological

TABLE 6.—*Salmonella isolations from 44 dogs before, during and after treatment, and in untreated controls.*

Group	Pretreatment			During treatment			Post-treatment		
	Examinations	Positive		Examinations	Positive		Examinations	Positive	
		Number	Percent		Number	Percent		Number	Percent
Treated									
Aureomycin	5	4	80	35	35	100	20	17	85
Chloromycetin	6	6	100	40	36	90	24	19	79
Terramycin	6	6	100	42	30	71	24	18	75
Pectadiazine	6	5	83	41	40	98	24	13	54
Total treated	23	21	91	158	141	89	92	67	73
Controls	21	21	100	146	143	98	84	67	80
				Isolations					
Treated									
Aureomycin	5	7	140	35	40	114	20	21	105
Chloromycetin	6	11	183	40	51	128	24	22	92
Terramycin	6	9	150	42	41	98	24	21	88
Pectadiazine	6	8	133	41	51	124	24	18	75
Total treated	23	35	152	158	183	116	92	82	89
Controls	21	33	157	146	195	134	84	84	100

houses who supplied anitibiotics and of veterinarians who were anxious to try them in cases of salmonellosis with acute diarrheas, we observed the clinical and bacteriological response in a small series of ill animals. It proved impracticable to treat alternate cases, and it can be stated only that the antibiotics appeared to have little, if any, effect on the course of the disease or on the cultural findings.

It was reported above that in kennel *H* on September 25th multiple salmonella infections were found to be widely prevalent with no apparent symptoms of illness. Therapeutic trials began on September 29th. Twenty-one dogs were held as untreated controls, five were given aureomycin and six each chloromycetin, terramycin, and sulfadiazine. The animals varied in weight from 50 to 68 pounds. The antibiotics were given in an initial dosage of 750 mg and a maintenance dose of 250 mg morning and evening. The sulfonamide was given in a compound commonly used in veterinary practice, pectadiazine; the sulfadiazine dosage was 0.8 g three times daily. Each drug was given for seven days. The response was measured by daily cultural examinations while therapy was being given and twice weekly thereafter for two weeks. The observations are summarized in table 6. The treatments used had minor, if any, effect on the infections as measured by the percentage of cultures positive or the total number of salmonella isolations.

In these survey examinations 35 types of salmonella were found, 31 from Jacksonville and 25 from Miami. The distribution of these by frequency of occurrence was shown in table 2 of the preceding paper in this series. The similarity in the distribution of the types in the two areas was notable, rather than the minor differences.

The prevalence rates for the four commonest types of salmonella in Jacksonville kennels was computed. It was apparent that there were kennel outbreaks with different types. For example, *S. rubislaw* was a common infection in two kennels but was not isolated in 9 of the 15 kennels.

Simultaneous infections with different types of salmonella were discovered. It was the usual practice to pick from two to four colonies per specimen, and a heavy infection could readily obscure a light one. In some instances two types were found in one Kligler tube inoculated from what was picked as one colony. The most frequent type, *S.*

anatum, occurred in 150 double combinations. The greatest number of *S. anatum* combinations, 78, occurred with *S. derby*, the second most frequent type. Other combinations with *S. anatum* were found with decreasing frequency. *S. derby* occurred in 96 combinations, *S. rubislaw* in 50, and *S. oranienburg* in 39. As the frequency of the type decreased, its appearance in combination was more erratic. There were 21 triple arrangements involving 13 types, *S. anatum* occurring in 18 of these. One instance of a simultaneous infection with *S. anatum*, *S. rubislaw*, *S. oranienburg* and *S. bredeney* was noted.

The occurrence of double combinations involving *S. anatum*, *S. derby*, *S. rubislaw*, and *S. oranienburg* was checked by the chi-square test, and it was found that these occurred more frequently than would be expected by chance alone. The observed double combinations were about twice the expected number.

SUMMARY

1. The nature of the greyhound industry in Florida is described.

2. All animals in kennels were examined by survey cultures. In Jacksonville, of 1602 cultures, 697 (43.6%) were positive with 878 isolations of salmonella; and in Miami, of 946 samples, 233 (24.6%) were positive with 251 isolations. An additional 524 cultures were taken during a therapeutic trial on 44 dogs; 460 (87.8%) were positive with 612 isolations. In all, 1518 greyhounds were tested. There were 3072 cultures, which yielded 1390 positive specimens and 1741 isolations of salmonella.

3. The proportion of animals positive increased with the number of examinations, reaching 98% in those cultured five or more times.

4. Annual minimal attack rates based on monthly cultural findings were in excess of 600 per 100 dogs per annum or an infection every two months. The true rate based on an intensive cultural study of one group appeared to be in the order of three infections per dog per month.

5. There was a seasonal variation, with higher rates in the fall and early winter.

6. The cleaner kennels had lower rates, but the difference was not marked.

7. Thirty-five types of salmonella were found, with multiple simultaneous infections detected more frequently than would be expected by chance alone.

8. Therapeutic trials failed to reveal any significant modification of the infections by any of three antibiotics or sulfadiazine.

SALMONELLOSIS IN DOGS

III. PREVALENCE IN DOGS IN VETERINARY HOSPITALS, POUNDS AND BOARDING KENNELS

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In veterinary hospitals, pounds and boarding kennels there is a rapidly changing animal population. With few exceptions, each dog could be cultured only once, and none were followed for more than short periods. All hospitals in Jacksonville were visited repeatedly. In one instance 22 cultural surveys were obtained in one hospital during a period of 15 months. The initial survey was taken without knowledge or consideration of prevailing illnesses or infection and may be accepted as a random sampling of dogs in veterinary hospitals. Repeat surveys were sought more consistently and repeatedly where the prevalence of salmonellosis was high. It was the practice to culture all animals which could be handled safely, omitting only recent surgical cases and vicious dogs. The same general practices were followed in the examinations in pounds and boarding kennels. All specimens were collected by rectal swab, and the bacteriological procedures were those described in the first paper of this series.

The findings on cultures taken in different veterinary hospitals are shown in table 1. On the first and random sampling there were 503 cultures, of which 76 (15.1%) were positive for salmonella. On the 1945 tests in subsequent surveys, 449 (23.1%) were positive. From hospital to hospital and within the same hospital, the proportion of positive findings varied markedly. The two hospitals in Jacksonville (no. 10 and 14) with low rates on the initial tests had high rates on the repeat examinations.

The results on dogs in pounds are given in table 2. The prevalence was comparatively low, particularly in Jacksonville. These bacteriological studies on dogs began in the Jacksonville City Pound, and the percent of positives may not be wholly comparable to that found elsewhere. The early studies from this source yielded 2.6% positive from 303 specimens. One year later 4.2% of 320 dogs were positive. Certainly, it is to be noted that excluding the findings from this pound, 18% of the pound dogs were found positive for salmonella.

The boarding kennels accepted only healthy dogs for care. The animals were kept in individual kennels but in the exercise pens had close contact with other dogs. Of 126 dogs tested in four boarding kennels, 21 (16.6%) were positive for salmonella, as shown in table 3.

There were 41 types of salmonella isolated in all. For 12 of these types, there were 25 or more isolations each, but for 22 there were less than 10 isolations each. The most commonly encountered were *S. anatum*, *S. manhattan*, *S. senftenberg*, *S. montevideo* and *S. typhimurium*.

There was clear evidence of localized outbreaks in some of the hospitals. Prior to August, 1949 in one hospital (no. 7), all animals had been tested twice. There was one isolation of *S. typhimurium* only. During August, 1949, there were two surveys, and 6 and 20 of the 48 and 51 dogs examined were

Received for publication October 24, 1951.

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TABLE 1.—*Cultural surveys for salmonellosis on dogs in veterinary hospitals in Florida.*

Veterinary hospital	Initial examinations			Repeat examinations			Total			Salmonella isolations
	Specimens	Positive		Specimens	Positive		Specimens	Positive		
		Number	Percent		Number	Percent		Number	Percent	
1	77	1	1.2				77	1	1.2	1
2	16	1	6.1				16	1	6.1	1
3	20	3	15.0	51	5	9.8	71	8	11.4	8
4	15	4	20.0	108	10	9.2	123	14	11.4	19
5	64	8	12.2				64	8	12.2	8
6	8	1	12.5				8	1	12.5	1
7	60	4	6.6	291	53	18.2	351	57	16.2	59
8	36	10	27.7	95	14	14.7	131	24	18.3	24
9	15	10	66.6	72	8	11.1	87	18	20.6	24
10	19	0	0	24	10	41.7	43	10	23.2	10
11	47	12	27.6				47	12	27.6	14
12	36	12	33.3	952	228	23.9	988	240	28.1	259
13	53	4	7.6	88	34	38.6	130	39	29.2	45
14	28	1	3.5	264	87	36.7	292	88	30.1	114
15	10	5	50.0				10	5	50.0	6
Totals	503	76	15.1	1945	449	23.07	2438	526	21.5	593

positive for *S. typhimurium*. There were two surveys in September; in each, 50 dogs were tested and each time three were positive for *S. typhimurium*. On one survey of another hospital (no. 9) 15 of 29 dogs were positive for salmonella, and 13 were *S. madelia*. One year previously *S. madelia* was isolated from a cat in this hospital. No specimens were obtained during the intervening period. In another hospital (no. 12) on different occasions there were 12 positives, of which 10 were *S. poona*, 13 positives with 9 *S. worthington*, and 26 positives with 18 *S. tennessee*. By contrast, in some a wide variety of types was found, each present in small numbers only. On three successive surveys in one hospital (no. 14) there were 28 positives with 11 types, 15 positives with 9 types and 14 positives with 10 types. In all, 16 types were found on the three surveys conducted within 21 days.

TABLE 2.—*Cultural surveys for salmonellosis on dogs in pounds in Florida.*

Pound	Specimens examined	Positive		Salmonella isolations
		Number	Percent	
1	623	21	3.3	21
2	63	3	4.7	3
3	24	2	8.3	2
4	74	11	14.8	24
5	13	2	15.4	2
6	10	3	30.0	3
7	88	29	32.9	32
Totals	895	71	7.8	87

TABLE 3.—*Cultural surveys for salmonellosis on dogs in boarding kennels in Florida.*

Boarding kennel	Specimens examined	Positive		Salmonella isolations
		Number	Percent	
1	53	5	9.4	5
2	40	4	10.0	4
3	5	1	20.0	1
4	28	11	39.2	11
Totals	126	21	16.6	21

There were 701 isolations of salmonella from the 618 positive specimens. Four types were found in one specimen, three in each of ten specimens, and from each of 60 specimens there were double isolations.

Bacteriological surveys of the environment were also conducted in an effort to discover the possible source or sources of contamination. Swabs were taken from the food, food preparation utensils, watering pans, cages, animal runs and from the dust pan. All swabs were inoculated to enrichment broth and examined in the manner used for fecal specimens. In hospital #13, four of the five swabs from food yielded salmonella, one of the ten swabs from food preparation utensils was positive, and a swab from the dust pan was also positive. Of the six positives, three were *S. anatum* and three were *S. montevideo*. Eight swabs of animal cages and runs, three of watering pans and twelve others were

all negative. On a repeat survey one week later *S. anatum* was again isolated from animal food in the deep freeze. Bacteriologic surveys in this hospital had yielded the following findings. In the summer of 1949 there were four and three positives in the 52 and 28 dogs tested in this hospital. But in July, 1950, of 40 dogs tested 28 were positive. Including multiple infections, 25 *S. anatum*, four *S. derby*, three *S. senftenberg* and one *S. montevideo* were isolated at this time. The swab testing of the environment was undertaken as soon as it was known that there was this unusual prevalence. It was ascertained that animal liver was the basic food in this hospital, that it was obtained locally and included those rejected for human consumption. The livers were ground and were not heated at a degree or for a time adequate to kill salmonella. It was subsequently found that reject livers as handled in abattoirs are commonly positive for salmonella.

In a second hospital (no. 12), *S. manhattan* had not been isolated during 20 surveys in the 14 preceding months; but in early September, 1950, of 45 cultures taken, 36 were positive for *S. manhattan*, and one week later of the 39 dogs tested, 25 were positive for this type. In the interval between these last two surveys, 34 swab tests of the physical environment were taken and four were positive for *S. manhattan*. Prepared food in a bucket ready for feeding and remnants of food in an individual feeding pan were both positive. The other two positives came from the floor of the exercise pens. According to the veterinarian, commercial animal foods were used exclusively, so the contamination of food in this instance may have been from extraneous sources.

Comparable bacteriological studies of the environment of other hospitals were not undertaken.

SUMMARY

1. The bacteriological findings on 3459 specimens from dogs in 15 veterinary hospitals, 7 pounds and 4 boarding kennels in 8 different cities of Florida are presented.

2. Of 2438 specimens from hospitalized dogs, 525 (21.5%) yielded salmonella, as did 71 (7.8%) of 895 impounded dogs and 21 (16.6%) of 126 animals in boarding kennels.

3. There were 701 isolations of salmonella, representing 41 types from 618 positive specimens.

4. The proportion positive in different hospitals, pounds and boarding kennels varied markedly.

5. Localized outbreaks were observed in several hospitals.

6. Bacteriological surveys of the environment in two hospitals yielded the same salmonella types as those previously found in the dogs from prepared food, utensils, fresh livers, a dust pan and the floor of an exercise pen.

SALMONELLOSIS IN DOGS

IV. PREVALENCE IN NORMAL DOGS AND THEIR CONTACTS

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Observations on the occurrence of salmonellosis in normal dogs living in their usual environment are notably rare. Watt and DeCapito[1] cultured 1156 family dogs in Hidalgo County, Texas and found 39 (3.4%) positive for salmonella. Other than this work, reported studied on dogs have given attention to findings on sick animals, those in contact with ill humans or those being cared for in pounds, kennels or animal hospitals. During the studies reported here 1626 family dogs have been examined bacteriologically, 244 (15.1%) were positive for salmonella (table 1).

The culture techniques used were those described in the first paper of this series. The only unusual feature of this study was the difficulty in obtaining the animals for culture.

A field worker was first assigned to the veterinary hospitals to obtain cultures of dogs as they arrived for bathing, immunization, examination for surgical or skin disorders, or on entering the hospital as boarders. The relatively small numbers of animals contacted per day made this procedure very time consuming, but cultures were taken on 66 dogs in this manner and 13 (19.7%) were positive. The salmonella isolated were of nine different types. Two dogs owned by the same individual both yielded *S. bredeney* and *S. barielly*. Another dog yielded both *S. canoga* and *S. cubana*. The remaining three *S. cubana* cultures isolated from normal dogs during the entire study were from animals which had been seen in the same hospital where this infection was widely prevalent among in-patients. The 66 cultures were taken in four different hospitals on ten days scattered throughout a four-month period. The dogs examined were well cared for and came from families above the average economically.

The most profitable source of specimens from normal dogs was in "rabies clinics." It is the practice of veterinarians in this state to provide rabies vaccination in clinics arranged at convenient locations. A member of our group accompanied the veterinarians to 22 such clinics in 1950 and to eight in 1951. These were held in 11 counties of the state. A total of 1385 dogs were cultured with 194 (14.0%) positive for salmonella. The proportion found positive in the different clinics varied from 1.9 to 41.0% in 1950 and from 5.5 to 22.8% in 1951. The 41 types of salmonella found are shown according to clinics in table 2. The variety and wide distribution of types is notable.

The highest proportion of positives (41%) was found in the Homosassa Rabies Clinic. A more detailed study was subsequently undertaken in this community. The rabies clinic was held on July 15, 1950, and revealed 16 positive animals. On July 25, the com-

Received for publication October 24, 1951.

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1. Watt, J. and DeCapito, T. 1950, Frequency and distribution of *Salmonella* types isolated from man and animals in Hidalgo County, Texas Am. J. Hyg. 51: 343–352.

TABLE 1.—*Salmonellosis in normal dogs.*

Year	Source of dogs examined	Specimens			Salmonella isolations	
		Examined	Positive		Number	Per 100 specimens
			Number	Percent		
1950	Outpatients in veterinary hospitals	66	13	19.7	16	24.3
	Rabies immunization clinics	917	143	15.5	149	16.2
	Homosassa* surveys	112	26	23.2	26	23.2
	Rabies clinics follow-ups	63	11	17.5	11	17.5
1951	Rabies immunization clinics	468	51	10.9	55	11.8
Totals		1626	244	15.0	257	15.8

* Florida rabies clinic.

munity was revisited and cultures were taken on 12 of the positive animals then available. Of these, seven were negative, three yielded the same type of salmonella isolated ten days earlier, and two yielded a type different from that found on the previous culture. Beginning August 8, a detailed bac-

TABLE 2.—*Distribution of salmonella types among normal dogs and their contacts.*

	Salmonella types	Rabies clinics																								Totals
		1950																						1951		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	8	
B	*S. paratyphi B.*																				1		1	2	4
	S. saint paul													2							1	2	2	2	9
	S. san diego						1																	2	3
	S. derby								1					1		1		1*							4
	S. typhimurium							2	1									1						2	6
	S. bredeney																					2	3	6	11
C_1	*S. norwich*																							1	1
	S. montevideo						1				1													2	4
	S. oranienburg										1								2					4	7
	S. bareilly								1						1								1	1	4
	S. hartford										1*												1	2	4
	S. tennessee																							2	2
C_2	*S. newport*			1						1			1								2	2		2	9
	S. muenchen					1				1*		1		1										5	9
	S. manhattan																				1			1	2
	S. bonariensis																							1	1
	S. litchfield														1*			1				2		2	6
	S. tallahassee									1	1											2†	5		9
	S. kentucky														2										2
D	*S. miami*						1	1	1	1	2*	2		1		1	1		3	2	1	1		1	19
	S. javiana																				2	2†		1	5
E_1	*S. anatum*		1		1				1	2	1							1*			1	3		5	16
	S. give								1										1						2
E_2	*S. newington*																		1						1
	S. newbrunswick									1															1
E_3	*S. senftenberg*															1								1	2
F	*S. rubislaw*									4	1	1	2		2				1		1			2	14
	S. luciana		1				2																		3
G	*S. worthington*	1																	1					1	3
	S. poona										1											1			2
H	*S. florida*									3*				1	1	1						3			9
	S. madelia										1										1			1	3
	S. carrau									1	1			1							2	1		1	7
	S. homosassa					1													2				2		5
I	*S. gaminara*														1*						1		1	2	5
FG	*S. cerro*						1															1		1	3
	S. inverness						1																		1
	S. minnesota								1															2	3
	S. pomona						1															2			3
Totals		1	2	1	1	2	8	3	7	15	11	4	3	7	8	4	1	4	11	2	14	24	16	55	204
Percent positive		2	4	4	5	6	9	10	12	18	15	15	17	19	20	20	20	22	21	25	26	35	41	11	

* Two types isolated from same animal.
† Both types isolated from same animal in two instances.

TABLE 3.—*Cultural findings for salmonella in humans and their animals.*

Cultures taken	Homosassa*			Follow-up of other rabies clinics		
	Specimens examined	Positive		Specimens examined	Positive	
		Number	Percent		Number	Percent
Humans	385	2	0.5	113	3	2.7
Dogs positive in rabies clinics	22	8	36.4	39	9	23.2
Other dogs	90	18	20.0	24	3	12.5
Cats	73	9	12.3	18	2	11.1
Chickens	46	1	2.1	19	0	0
Other fowl	7	0	0	7	0	0
Hogs	9	0	0	3	0	0
Cattle	5	0	0	3	0	0
Horses	4	0	0	3	0	0
Goats	2	0	0	0	0	0
Rabbits	2	0	0	4	0	0
Racoon	1	0	0	0	0	0
Turtle	1	1	100	0	0	0

* Florida rabies clinic.

teriological study of all families and animals in the area was started. Rectal swabs were taken on children and on available animals; fecal specimens were requested from the adult humans. The findings for this community are summarized in the first columns of table 3. Ten dogs positive at the time of the rabies clinic were recultured in this general survey, and two were found to harbor the same type of salmonella which was isolated one month previously.

Elsewhere there was a limited follow-up of families where one or more dogs were found positive during the rabies clinic. These studies were started one month or more after the clinic. The findings are summarized in the second part of table 3.

The striking feature throughout all these studies of normal dogs was the variety of salmonella types identified. In Homosassa, for example, one dog cultured three times yielded on July 15, *S. homosassa*, on July 25, *S. poona*, and August 8, *S. saint paul*. At the rabies clinic the dog of another family was positive for *S. tallahassee*. Later cultures yielded *S. saint paul* from this dog, *S. san diego* from another dog and *S. hartford* from chickens on the one farm. In another community three dogs in each of two families were simultaneously positive, involving six salmonella types. There were six instances of two infections with the same salmonella type in the humans or animals of a household, but there were 21 instances in which two, three or four types of salmonella were isolated in the one household and its animal environment. These findings suggest numerous reservoirs of salmonella infections.

Data did indicate, however, that there are variations in the prevalence of types in different communities. *S. tallahassee* was found in few communities, and 15 of the total of 20 isolations were from Homosassa. Eleven of the 15 *S. saint paul* isolations, six of the seven *S. hartford* and five of the ten *S. bredeney* were from this one community. By contrast, *S. miami*, *S. anatum* and *S. rubislaw* were found commonly elsewhere, but not in Homosassa.

Salmonella infections of dogs appeared to persist for a short period. Of 49 positive animals which were cultured from four to six weeks after the rabies clinic only five yielded the same salmonella type found on the initial culture. However, within this period of observation seven of these positive dogs were found to have acquired a second and in some instances a third type.

Salmonella were isolated from 11 (12.1%) of 91 cats examined, from one of

65 chickens and from the one turtle tested. The other 50 animals or birds cultured were negative.

No evidence was obtained on which to judge the epidemiological significance to man of salmonella infections of normal family dogs. Five humans positive for salmonella were found in the follow-up surveys; in three there were no other positive humans or animals in the household. In one the human was positive for *S. oranienburg*, while his dog was positive for *S. miami*, and five weeks earlier the dog was positive for *S. muenchen*. One of the two humans positive in Homosassa yielded *S. saint paul*, a type common in the domestic pets of that community.

SUMMARY

1. Specimens for bacteriological examination on normal family dogs were procured through community surveys, rabies immunization clinics and in veterinary hospitals (using dogs brought for immunization, bathing, boarding and surgical conditions).

2. A total of 1626 normal dogs were cultured; 244 (15.0%) were positive for salmonella.

3. The 41 types of salmonella encountered were widely distributed, but variations in prevalence were found.

4. There was no secure evidence as to the epidemiological significance to man of salmonella infections in the normal family dog.

THE BACTERICIDAL ACTION OF DOG BLOOD AGAINST *BRUCELLA SUIS*

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As part of a program concerned with host factors in natural resistance to infection, the bactericidal activity of blood of various species was studied employing avirulent and virulent strains of brucella. Reports from several laboratories[1–4] suggest that bovine serum and plasma possess greater bactericidal activity than whole blood against *Brucella abortus*. The present paper describes the bactericidal action of the blood of the dog, an animal of low susceptibility to brucellosis, against a virulent strain of *Brucella suis*. In contrast to the reports on bovine blood, it was found that whole dog blood was bactericidal while dog plasma or blood cells were not.

METHODS

The bactericidal effect of whole blood, plasma, and buffy coat, or red blood cells in plasma, was tested against *Br. suis* PSIII, a virulent, smooth strain. Fresh heparinized blood and plasma were employed unless otherwise stated.

Buffy coat was prepared in the following manner: Blood was centrifuged at 3000 r.p.m. for one hour and the plasma removed. Since the lower portion of the sediment was almost free of white blood cells, approximately three-fourths of the lower portion of the sediment was withdrawn by pipette. The top part of the sediment was mixed with 0.5 volume of plasma and recentrifuged in smaller tubes. After repeated removal of red blood cells, the remainder of the sediment contained between 50,000 and 200,000 white blood cells per cmm. The numbers of white and red blood cells in this material were determined. White or red blood cell counts were adjusted by adding or removing plasma or adding red cell sediment.

Red blood cells were obtained from the bottom layer of sediment of the centrifuged blood and diluted with plasma so that the red blood cell count was similar to that of the buffy coat. This material contained from 0 to 350 white blood cells per cmm.

In certain experiments, blood cells were washed with Krebs solution[5] containing 3% gelatin. This Krebs solution contained the same concentrations of K, Ca, Mg, PO_4, HCO_3, and SO_4 as an ultrafiltrate of dog serum. More Na ion was present than that in ultrafiltrate. Gelatin-Knox P-20 "for intravenous clinical use" contained 6% gelatin in 0.9% NaCl and was diluted with an equal volume of 0.9% NaCl. To 100 parts of the 3% gelatin in 0.9% NaCl were added 4 parts of 0.154 *M* KCl, 3 parts of 0.11 *M* $CaCl_2$, 1 part of 0.154 *M* KH_2PO_4, 1 part of 0.154 *M* $MgSO_4$, and 4.6 parts of 0.592 *M* $NaHCO_3$. This solution will be referred to as Krebs-gelatin solution.

Cultures of *Br. suis* PSIII grown 24 hours on tryptose agar slants were washed and suspended to 1×10^9 organisms per ml in tryptose saline by matching a cholesterol standard. Decimal dilutions were made in 0.9% NaCl, and the number of viable organisms was determined by the dilution plate count method.

Tubes containing bacteria were placed in a rotator[6] which turned 24 times each minute. The importance of agitating bacteria and other particles exposed to phagocytosis has been repeatedly emphasized.[7,8] Blood or its various constituents were added to the bacteria at the be-

Received for publication November 8, 1951.

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TABLE 1.—*Percent survival of* Br. suis *PSIII in dog blood and its constituents. Effects of temperature, time, intervals of addition of fresh constituents and total volume.*

Material	Temperature (C)	Number of experiments	Duration (hrs.)	Intervals between additions (min.)	Volume in ml	Mean ± S.E. percent survival	Standard deviation
Whole blood	20*	17	2	30	4.0	5.8± 0.86	3.6
	20*	10	4	30	8.0	10.6± 2.02	6.5
	37	4	2	30	2.0	6.2± 1.78	3.6
	37	14	2	30	4.0	4.3± 0.44	1.6
	37	16	2	30	8.0	5.4± 0.65	2.7
	37	19	4	30	8.0	5.3± 1.11	4.9
	37	6	2	60	2.0	3.8± 1.26	2.9
	37	2	4	15	16.0	4.8	
Citrated blood plasma	37	11	2	30	4.0	79.2± 9.34	31.1
	20*	5	2	30	1.8	112.2±25.95	58.7
	20*	5	2	60	1.0	97.6±20.61	46.2
	37	5	2	30	1.8	100.8±23.43	52.8
	37	6	2	60	1.0	70.7±13.35	32.8
Citrated plasma	37	10	2	30	2.0	63.1± 7.42	23.8
Buffy coat	20*	8	2	60	2.0	5.1± 1.04	3.0
	20*	2	2	120	1.0	8.1	
	37	12	2	60	2.0	6.8± 1.33	4.8
	37	5	2	120	1.0	7.5± 3.26	7.5
Red blood cells	37	7	2	60	2.0	35.9±11.12	29.9

* Room temperature.

ginning and at intervals throughout the two or four hours of observation. When comparing the action of blood constituents with the action of blood, the quantities employed were similar, i.e., the quantity of plasma employed was derived from the hematocrit and therefore was that contained in a unit of blood. Departures from this plan are indicated in the text. At the end of individual experiments, the number of organisms remaining in each tube was determined by the dilution plate count method. For convenience of presentation, the percentage of surviving organisms was recorded.

RESULTS

Various factors which might influence bactericidal properties of blood constituents were investigated, namely, number of organisms, temperature, duration of exposure, volume of fresh blood constituents, interval between additions of fresh constituents, and anticoagulants. Bactericidal effects were studied at 37 C and at room temperature (20 to 27 C) for two and four hours in 2, 4, or 8 ml total volume, and with fresh material added at 30- or 60-minute intervals; heparinized and citrated blood were compared.

Table 1 summarizes results of the experiments above.

Effect of number of Br. suis *PSIII on percentage of surviving organisms in blood*

To determine the number of organisms suitable for a standard bactericidal test, different numbers of *Br. suis* PSIII were mixed with 2 ml of blood for 2 hours at 37 C. The percentage of surviving organisms was similar when the initial number of bacteria ranged from 5×10^6 to 5×10^8. The percentage increased, however, when the bacteria were more than 10^9. With an original count of about 5×10^6, approximately 5% of *Br. suis* PSIII survived in 78 tests. With 5×10^7 and 5×10^8, the percentages of survival were 5.5 and 4.1, respectively. With counts of 10^9, 10^{10}, 10^{11}, and 10^{12} (in 8 to 55 experiments with each number), the mean percentages of survival and their standard errors were 14.8 ± 3.9, 35.6 ± 5.7, 63.1 ± 8.7, and 116 ± 20.8, respectively. Statistical analysis revealed that there was no detectable bactericidal action when the number of brucella was 10^{11} or greater. Since optimal bactericidal effects occurred with 5×10^6 to 5×10^8 bacteria, the former number was se-

lected for subsequent tests, viz., 5×10^6 organisms suspended in 0.05 ml of saline.

Effects of temperature on bactericidal action of blood and its constituents

Table 2 shows the mean percentage survival and its standard error of *Br. suis* PSIII, the differences between the means, and the approximate probability that the differences were due to chance at room temperature and at 37 C. During the two-hour mixing period, heparinized blood was added in 1 ml volumes at 30-minute intervals. Plasma of the same blood was added in 0.45 ml volumes at similar intervals for the same time. The plasma volume was equivalent to that contained in the blood. Buffy coat was added in 1 ml volumes at 60-minute intervals and rotated for two hours.

When *Br. suis* PSIII was mixed with blood for two hours, (series A1 and 2 at room temperature) the mean percentage surviving was 5.8 ± 0.86. At 37 C, under similar conditions, the mean percent surviving was 4.3 ± 0.44. The difference was not statistically significant.

With plasma, series B1 and 2, at room temperature the mean percent survival in five experiments was 112.2 ±25.95. Under similar conditions at 37 C the mean survival was 100.8 $\pm23.43\%$. Under these conditions heparinized dog plasma had no bactericidal action.

In series C1 and 2, buffy coat was mixed with *Br. suis* PSIII at room temperature and at 37 C for two hours. The percentages of surviving organisms at the lower temperature were 5.1 ± 1.04 and at 37 C 6.8 ± 1.33. The mean difference in survival was not statistically significant.

Effects of duration of exposure on survival of Br. suis *PSIII*

Extension of the period of incubation at 37 C did not significantly increase the bactericidal effect. The mean survival after two hours of mixing was 5.4 ± 0.65, and after four hours, 5.3 $\pm1.11\%$ (table 1, lines 5 and 6).

The experiments at room temperature were not strictly comparable with those at 37 C, for at room temperature the total quantity of blood for the two-hour experiments was 4 ml, while for the four-hour period the total quantity was 8 ml. Blood was added in 1 ml volumes at 30-minute intervals. After two hours at room temperature, the mean percent survival was 5.8 ± 0.86, after four hours, 10.6 ± 2.02 (table 1, lines 1 and 2). The mean difference of 4.8 ± 2.2 was probably significant, with a P value of 4%. However, the results cannot be attributed to differences in volume. As will be seen below, volume was not a determining factor in the bactericidal action of blood. Although

TABLE 2.—*Percent survival of* Br. suis *PSIII for two hours in dog blood and its constituents. Effects at room temperature and 37 C.*

Constituent	Temperature (C)	Number of experiments	Percent survival		Approximate probability
			Mean ± S.E.	Difference ± S.E.	
A_1 blood	20–27*	17	5.8± 0.86	A_1-A_2	
A_2	37	14	4.3± 0.44	1.5±0.98	14%—NS
B_1 plasma	20–27*	5	112.2±25.95	B_1-B_2	
B_2	37	5	100.8±23.43	11.4±37.96	77%—NS
C_1 buffy coat	20–27*	8	5.1± 1.04	C_1-C_2	
C_2	37	12	6.8± 1.33	1.7± 1.78	35%—NS

NS = not significant.
* Room temperature.

bactericidal action was the same at both temperatures for two hours, thereafter bactericidal action at room temperature lagged somewhat behind bacterial growth.

The effect of the addition of fresh blood at various intervals to the mixture with Br. suis *PSII*

Comparison was made of the bactericidal action of blood at 37 C in systems to which 0.5 and 1 ml fresh blood were added at intervals of 30 and 60 minutes, respectively, during two hours of mixing. The final volume in both series was 2 ml (table 1, lines 3 and 7). Experiments with 30-minute intervals between additions of blood showed a mean percentage survival of 6.2 ± 1.78, not significantly different from the other, 3.8 ± 1.26.

The effect of volume of blood on bactericidal action against Br. suis *at 37 C*

The total volume of blood added to *Br. suis* PSIII at 30-minute intervals over a two-hour period was 2 ml, 4 ml, and 8 ml, respectively (table 1). The mean percentages of survival were similar,—6.2 ± 1.78, 4.3 ± 0.44, and 5.4 ± 0.65, respectively.

With four-hour periods, 1 ml fresh blood was added at 30- and 15-minute intervals to final volumes of 8 and 16 ml, respectively. With 8 ml of blood the mean percent survival was 5.3 ± 1.11, not significantly different from 4.8 with 16 ml. In spite of only two experiments with 16 ml of blood, it was apparent that volume of blood produced no effect on bactericidal action.

Comparison between heparinized blood and plasma on the bactericidal action against Br. suis *PSIII*

At room temperature, heparinized blood was mixed with *Br. suis* PSIII for two hours, adding 1 ml every 30 minutes to a final volume of 4 ml. The mean percentage survival was 5.8 ± 0.86 (table 3). Plasma was added in 0.45 ml quantities at 30-minute intervals to a total of 1.8 ml, an amount equivalent to that contained in blood with which it was compared. The mean percentage survival was 112.2 ± 25.95. Similarly, at 37 C the mean percent survival in blood was 4.3 ± 0.44. and in plasma 100.8 ± 23.43. Dog plasma showed no bactericidal properties against *Br. suis* PSIII in two hours.

Comparison between bactericidal action of citrated blood and plasma

Most of the reports[1–4] dealing with bactericidal action of blood and plasma have been carried out with citrated blood. In the present experiments, citrate was avoided as an anticoagulant, because citrate is known to interfere with phagocytosis.[7,8] Having found that dog blood cells were essential for the bactericidal action against *Br. suis* PSIII in heparinized preparations, it was of interest to study the effect of citrate upon this reaction. Sodium citrate was added to blood in a concen-

TABLE 3.—*Comparison of two-hour bactericidal action of heparinized dog blood and of plasma against* Br. suis *PSIII at room temperature and 37 C.*

Material	Temperature (C)	Number of experiments	Percent survival		Approximate probability
			Mean ± S.E.	Difference ± S.E.	
Blood	20–27*	17	5.8± 0.86		
Plasma	20–27*	5	112.2±25.95	106.4±25.95	<0.1%
Blood	37	14	4.3± 0.44		
Plasma	37	5	100.8±23.43	96.5±23.43	<0.1%

* Room temperature.

TABLE 4.—*Comparison between citrated and heparinized blood and plasma on bactericidal action against* Br. suis *PSIII at 37 C for two hours.*

Material	Number of observations	Volume in ml	Percent survival		Approximate probability
			Mean ± S.E.	Difference ± S.E.	
a. Heparin blood	14	4.0	4.3 ± 0.44	(d–a) 58.8 ± 7.42	<0.1%
b. Citrate blood	11	4.0	79.2 ± 9.34	(a–b) 74.9 ± 9.34	<0.1%
c. Heparin plasma	5	1.8	100.8 ± 23.43	(c–b) 21.6 ± 25.21	40%—NS
d. Citrate plasma	10	2.0	63.1 ± 7.42	(c–d) 37.7 ± 24.62	16%—NS
				(b–d) 16.1 ± 11.86	20%—NS

NS = not significant.

tration of 0.8%, which inhibits phagocytosis.[8] Substitution of citrate for heparin was the only variable. The data in table 4 demonstrate the inhibition of bactericidal action by citrate. There were no significant differences between citrated blood, citrated plasma, and heparinized plasma.

The role of plasma in the bactericidal action of dog blood cells against Br. suis *PSIII*

Although dog plasma per se had no bactericidal action against *Br. suis* PSIII, experiments were designed to determine whether plasma played a role in the bactericidal activity of blood cells. Blood cells were separated from plasma by centrifugation. The cells were washed six times with tenfold volumes of Krebs-gelatin solution. Bactericidal action was compared between 1 ml washed blood cells suspended in either 1 ml of their original plasma or 1 ml of Krebs-gelatin solution. To these blood cell mixtures were added 5×10^6 *Br. suis* PSIII suspended in 0.05 ml of Krebs-gelatin solution. In order to equalize opportunities for phagocytosis in both systems, the bacteria added to the plasma-free blood cells were first sensitized with plasma of the donor dog and then freed of plasma by three washings, each with 5 ml of Krebs-gelatin solution. The final mixtures containing washed blood cells, liquid, and bacteria were rotated in tubes at 37 C for two hours. In 12 experiments recorded in table 5, the mean survival of *Br. suis* PSIII in washed blood cells and plasma was 4.9 ± 0.7%. In contrast, the survival of opsonized *Br. suis* PSIII in washed blood cells and Krebs-gelatin solution was 101 ± 11.4%. In spite of the great difference in bactericidal activity, phagocytosis was similar

TABLE 5.—*Effect of plasma on phagocytosis and bactericidal action of dog blood cells for opsonized brucella. Comparison of washed blood cells in homologous plasma and in Krebs-gelatin solution (KG).*

Percent neutrophils containing brucella		Percent survival of brucella	
Plasma	KG	Plasma	KG
39	22	2.6	89
64	94	1.8	41
49	20	10.1	106
96	86	4.1	56
56	68	2.1	142
97	88	7.2	73
82	40	9.0	96
82	51	4.2	127
100	64	4.3	140
95	86	5.5	152
91	46	5.4	140
98	100	2.8	46
Mean ± S.E.			
79 ± 6.2	64 ± 5.3	4.9 ± 0.7	101 ± 11.4

in both systems, showing a mean of neutrophils engulfing bacteria of 79 ± 6.2% for the former and 64 ± 5.3% for the latter system under standard conditions.[9] The mean difference of 15 ± 8.2 was not statistically significant. These experiments demonstrated that blood cells or plasma alone lacked bactericidal power against *Br. suis* PSIII. Bactericidal activity occurred only when blood cells and plasma were combined. Furthermore, phagocytosis was independent of bactericidal action.

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The role of leucocytes in bactericidal action against Br. suis *by dog blood*

It appears that bactericidal function of dog blood required the combined action of the cellular components and of plasma. In order to determine the cells involved in this reaction, tests were made with buffy coat and with suspensions of red blood cells free of white blood cells as described in *Methods*.

Bactericidal action of heparinized blood, buffy coat, or red blood cells mixed with plasma, and plasma alone against *Br. suis* PSIII at 37 C for two hours is recorded in table 1, lines 4, 17, 19, and 12, respectively. The bactericidal action of red blood cells plus plasma was much greater than that of plasma alone, but less than that of whole blood. Since all cellular suspensions contained the same amount of plasma and red blood cells, the differences in bactericidal action of suspensions with few white blood cells must be due to the difference in number of white blood cells. This effect of white blood cells was limited however, since buffy coat with 50,000 to 200,000 white blood cells per cmm had the same bactericidal action as whole blood.

DISCUSSION

The experiments indicated that the bactericidal power of dog blood required the combined activity of formed blood elements and plasma. That bactericidal action of dog leucocytes might be apparent rather than real was considered. If the leucocytes phagocytosed the bacteria, the bacteria might be intracellular aggregates. Upon dilution and plating, each aggregate might develop as a single colony. In these experiments, 5% of the organisms survived in dog blood. Assuming that all the intracellular bacteria were viable and that five colonies developed from each 100 growing bacteria, then individual leucocytes would contain approximately 20 bacteria. However, further considerations make this explanation untenable.

1. Direct smears of the mixture of 2 ml of blood and 5×10^6 bacteria disclosed no bacteria within leucocytes after the incubation period. Since *Br. suis* is very small, not much larger than a cytoplasmic granule, it is possible that individual bacilli within the leucocytes may have been missed. It is improbable, however, that collections or aggregates of this organism within or outside the leucocytes would have been overlooked.

2. When preparing bacterial plate counts, the suspensions of blood and bacteria were diluted 10^5 to 10^{12} times, and no intact blood cells were identified in the sediment. All cells were lysed. Bacterial counts therefore could not have been influenced by aggregated bacilli within leucocytes since all blood cells had been disrupted by the diluting liquid.

3. Experiments cited above demonstrated that phagocytosis of *Br. suis* showed no quantitative relationship to bactericidal activity.

If the bactericidal action of dog blood was limited to leucocytes, the number of organisms destroyed by individual leucocytes could be calculated. Assuming a leucocyte count of 10,000 per cmm, 2 ml would contain 2×10^7 leucocytes. Polymorphonuclear leucocytes were about 50% of the total white cells in dog blood, about 1×10^7 in 2 ml. In other experiments[9] mainly polymorphonuclear leucocytes phagocytosed *Br. suis*. In the present experiments, the percentage of bacteria destroyed by these blood cells was the same when the bacteria mixed with the blood varied between 5×10^6 to 5×10^8. When the number of bacteria was greater than 5×10^9 the percentage survival of *Br. suis* was greater. These observations suggest that for each polymorphonuclear

leucocyte present approximately 50 organisms were destroyed in two hours. This destruction was not due to phagocytosis since phagocytosis was independent of bactericidal activity.

Bactericidal action of blood or its constituents has been repeatedly demonstrated to vary with the species and with the strain of bacteria. About a half century ago, Pettersson[10] demonstrated bactericidal action of exudate leucocytes upon strains of "*B. proteus*." He distinguished between bactericidal action of exudate leucocytes and that of serum of the dog, cat, rabbit, and guinea pig. These observations were confirmed by many others, including Zinsser[11] and Manwaring,[12] who employed *S. typhosa* as the test organism, and Mackie et al,[13] who employed other organisms.

The reports dealing with bactericidal action of bovine blood against brucella indicated that activity was much greater in serum than in blood cells.[1–4] Irwin et al[1] found that citrated and defibrinated bovine blood had no bactericidal effect against *Br. suis*. Huddleson et al[4] found that the bactericidal action of bovine serum and bovine citrated plasma was much stronger against *Br. abortus* than against *Br. suis* or *Br. melitensis*. Khairat[14] found no bactericidal action of defibrinated human blood from one normal and from seven cases infected with *Br. abortus*. Hall and Spink[15] briefly stated that the bactericidal action of human serum was greater than that of whole blood and decreased in brucella bacteremia. In most of these studies blood cells were modified by irritant chemicals used to produce leucocytic exudates, by citrate which modified bactericidal action, or by defibrination which decreased leucocytes. Walsh and Harmsworth[16] reported that citrated blood had less bactericidal action than defibrinated blood.

The present experiments clearly showed bactericidal action of dog blood against *Br. suis* PSIII when formed elements and plasma were combined. Bactericidal action was evident in heparinized but not in citrated blood. Neither heparinized nor citrated plasma had significant bactericidal action against *Br. suis* PSIII. These observations suggest that dog blood differs from bovine or human blood with respect to bactericidal properties. However, since studies of bactericidal action in cattle and man were carried out with blood treated in a way which modified bactericidal functions in the dog, comparisons of bactericidal properties of blood from these species is not yet possible.

SUMMARY AND CONCLUSIONS

1. The bactericidal effects of dog whole blood, plasma, washed blood cells, buffy coat, and of red blood cells were compared under various conditions of temperature, time, additions of fresh blood, volume of blood, and number of *Brucella suis*.

2. Formed elements plus plasma were essential for the bactericidal action of heparinized dog blood against *Br. suis* PSIII. Leucocytes were more effective than red blood cells. Citrate abolished bactericidal action of dog blood. There was no significant bactericidal activity either in heparinized or citrated dog plasma or in washed blood cells.

3. Phagocytosis was independent of bactericidal activity.

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THE EFFECTS OF X IRRADIATION ON A NATURALLY OCCURRING ENDEMIC INFECTION

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A pulmonary infection in rats appeared at the Naval Radiological Defense Laboratory in a colony used for the study of biological effects of ionizing radiations. Despite vigorous efforts to eradicate the disease, the incidence grew to epidemic proportions, especially in groups of irradiated animals.

A similar infection has been reported from the Argonne National Laboratory in Chicago.[1] It was described as a chronic endemic disease which at one time became epidemic and reached an incidence of 95%, seriously interfering with the program of the Laboratory. The disease, thought to be caused by a gram-negative intracellular coccobacillus, did not respond to sulfa drugs or penicillin.

An epidemic pneumonia in rats has been reported also by the Radiology Section of Western Reserve University.[2] The magnitude of this epidemic reached such proportions that for six weeks all investigations in which rats were used had to be discontinued, for it was found that the afflicted animals were far more susceptible to injury from irradiation than were healthy rats.

The incidence of salmonellosis, which was "latent and endemic" in the rat colony at the University of Rochester, was found to increase among irradiated animals.[3] The incidence of the disease, or of its "activation," seemed to have been an increasing function of the dosage and, perhaps, of dose rate.

The effects of irradiation on the immune state have been the object of considerable study. Reference is made to reviews by Taliaferro and Taliaferro[4] and by Craddock and Lawrence[5] for extensive bibliographies on this subject. Recently the importance of infection

Received for publication December 13, 1951.

This work was made possible, in part, through funds provided by the Bureau of Medicine and Surgery, U. S. Navy Department, by the Office of Naval Research, and by the Atomic Energy Commission, under ONR contract with the University of California, Berkeley, California.

The opinions and assertions contained in this report are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large (Article 1252, U. S. Navy Regulations 1948).

The authors gratefully acknowledge the excellent technical assistance of Mr. A. C. Allen and the statistical analyses performed by Mr. H. H. Hechter.

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as a part of acute total body irradiation illness has received particular attention, and the efficacy of certain antibiotics in decreasing mortality among irradiated animals has been demonstrated.[6–9] Experimental data, together with the well-recognized prevalence of spontaneous infections among laboratory animals, emphatically point out the need for a more critical evaluation of infection, latent or active, as a complicating factor in radiobiological studies. There is a dearth of published information on this subject.

In the case reported in this paper, animals from the infected colony died following exposure to X-ray doses that were notably below those lethal for normal rats. This finding interfered seriously with the experimental work, since physiological changes and mortality are used as the criteria of response to irradiation. Thereafter the colony was not used as a source of animals for the scheduled research program of the Laboratory. In view of the above considerations, however, it seemed of value to obtain some measure of (a) the effects of X irradiation on the incidence and severity of the natural infection in the colony, and (b) the degree to which the infection influenced the validity of X-ray morbidity and mortality data.

DESCRIPTION OF THE DISEASE

The disease was manifested clinically by rales and frequently by weight loss. A fairly good correlation between these signs of the disease and gross lung pathology was observed. Not infrequently, however, animals that proved to have lung pathology appeared healthy, and vice versa. No gross lesions other than those of the lungs were discernible at autopsy. Throughout this study, "incidence of infection" refers to the incidence of gross pulmonary lesions as determined at autopsy.

No deaths were observed to result from the infection alone. However, during the course of this study, none of the animals in the colony attained an age greater than 200 days, so that the prognosis in later life is not known.

The mechanism of transmission of the disease was not determined. The disease did not respond to penicillin, and the use of triethylene glycol vapor in the animal rooms had no apparent effect on its course.

The gross pathology of the lungs could be divided into two distinct groups, which probably represented successive stages of the infection. One pathological entity consisted of brown areas of consolidation affecting an entire lobe of the lung or, more frequently, a part of one or more lobes. Another consisted of loculated areas of suppuration usually accompanying areas of pulmonary consolidation. The right upper and left lower lobes were affected most frequently. These were followed, in order, by the right middle, the left upper and the right lower lobes. Microscopically, the consolidated areas showed lesions of a chronic proliferative nature. A gram-negative, bipolar-staining coccobacillus was isolated from infected lungs of some animals from the colony. However, cultures of other rats with similar lesions did not yield this

6. Howland, J., Furth, F., Bennett, L., Coulter, M. and McDonnel, G. 1949, Studies on factors affecting the radiation syndrome. University of Rochester Report UR-94.
7. Miller, C. P., Hammond, C. W. and Tompkins, M. 1950, The incidence of bacteremia in mice subjected to total body X-radiation. Science **111**: 540–541.
8. Miller, C. P., Hammond, C. W. and Tompkins, M. 1950, Reduction of mortality from X-radiation by treatment with antibiotics. Science, **111**: 719–720.
9. Howland, J. W., Furth, F. and Coulter, M. 1950, Effect of aureomycin and antibiotics on whole body irradiation. Fed. Proc. **9**: 334.

organism. It was not possible to transmit the infection to normal rats by means of intranasal inoculation of the isolated coccobacillus. The infection was not transmitted to mice placed in the same cage with infected rats.

MATERIALS AND METHODS

The rat colony of the U. S. Naval Radiological Defense Laboratory was derived from breeding stock obtained from the Sprague-Dawley Farms in Madison, Wisconsin. All the rats were housed air-conditioned rooms where the temperature was maintained at 22 C. There was no cross circulation of air among the animal rooms. The first of the experiments reported here was carried out under these environmental conditions, and was designed to determine the dose-mortality response to X irradiation of presumably healthy rats in this Laboratory. For some weeks before this experiment was begun the infection had been endemic and the incidence low. This had been ascertained by inspection at weekly intervals of all animals in the colony. Rats suspected of infection (on the basis of rales and/or weight loss) were screened out, and the incidence of lung lesions in these animals was determined by autopsy.

X-ray mortality was exceptionally high in experiment 1. This fact, combined with aberrant results from other concurrent investigations, indicated that the infected colony should not be used for further regularly scheduled research. Therefore the colony was isolated in another building which had no air conditioning and where the temperature was maintained at 21 C to 26 C, and experiments 2 and 3, designed to study the relationship of irradiation to the disease, were conducted there. These studies were carried out three and five months later, respectively. Several weekly screenings, as described above, were performed prior to each experiment. Conditions of housing and the availability of animals necessitated some variation in the ages, sex and cage groupings of the rats used. These data, together with other pertinent factors, are included in the general outline of the experiments presented in table 1.

The irradiation factors were as follows: 240 to 250 KVP; 15 ma; tsd 100 cm, 0.5 mm Cu filter, and approximately 10 r per minute dose-rate.

In experiment 1, rats were exposed to graded doses of X radiation ranging from 650 to 1000 r, for the determination of the 30-day LD_{50}. A group of 20 rats served as controls. All animals that died during the experiment were autopsied and examined for gross indications of infection. The experiment was terminated at 30 days, when survivors and controls were sacrificed and examined for gross signs of infection.

The plan of experiment 2 was similar to that of

TABLE 1.—*Outline of experimental conditions and procedures.*

Exp. no.	Series	Treatment	Age at start of exp. (weeks)	Sex	Number per cage		Number of rats	X-ray doses (r)
					Before exp.	During exp.		
1		Irradiated	12–14	F	20*	5	37–40†	650, 700, 750, 800, 850, 900, 950, 1000
		Control	12–14	F	20	5	20	None
2		Irradiated	11–14	M	20–25	18	18†	20, 100, 200, 300, 400, 500, 600, 700
		Control	11–14	M	20–25	18	23 per sacrifice	None
3	1	Infected Irradiated	19–22	F	20–25	15	15†	300, 400, 500, 600, 700, 800, 900
	2	Infected Control	19–22	F	20–25	15	15–30 per sacrifice	None
	3	New stock Infected Irradiated	12–13	M	7	15	15†	300, 400, 500, 600, 700, 800, 900
	4	New stock‡ Infected Control	12–13	M	7	15	5 & 40 per sacrifice resp.	None
	5	New stock Irradiated	12–13	M	5	5	15†	500, 600, 700, 800, 900

* The larger groups of rats, 15 or more, were housed in cages 24×24×18 inches, the groups of 5 or 7 in cages 8×10×15 inches.
† Numbers given for irradiated rats indicate number per dose group.
‡ No known exposure except through caretaker.

the preceding experiment, except that a lower X-ray dosage range, 20 to 700 r, was employed in order to quantitate the combined effects of infection and radiation. A large number of non-irradiated controls was included to ascertain the incidence of infection in control animals throughout the 30-day period. Groups of 25 control rats were sacrificed and autopsied at 0, 8, 15 and 25 days.

Experiment 3 was designed to evaluate the combined effect of disease and X irradiation on healthy animals taken from an entirely different colony and newly exposed to the infection. For this purpose male rats were obtained directly from the Sprague-Dawley Farms. Adequate exposure of this healthy new rat colony to the infection was insured by placing two female rats showing gross indications of disease in each cage of 15 healthy males for a period of two weeks immediately before the start of the experiment. Autopsy at the end of this two-week period revealed an incidence of infection of 95% in these females, and at least one of these from each cage was heavily infected. Then experiment 3 was carried out as follows:

Series 1. Females from the infected colony (litter mates of the males used in experiment 2) subjected to doses of X radiation ranging from 300 to 900 r.

Series 2. Females, similar to those in series 1, which served as infection controls and were sacrificed at 0, 8, 14, 24 and 30 days.

Series 3. Sprague-Dawley males, newly exposed to the infection as described above, and given X-ray doses over the range of from 300 to 900 r.

Series 4. Sprague-Dawley males, newly exposed to the infection, and serving as infection controls. These animals were sacrificed at 24 and 30 days.

Series 5. Sprague-Dawley males maintained in separate clean quarters throughout the experiment, and exposed to X-ray doses ranging from 500 to 900 r. This series was intended to provide control data on X-ray mortality in "normal" rats. The caretaker tending the infected animals also cared for this group, without observing any special precautions with regard to isolation technique.

The mortality data obtained in these studies were analyzed by the probit method.[10]

RESULTS AND DISCUSSION

The results of these experiments are summarized in table 2. In the first experiment a 30-day LD_{50} could not be calculated because of the unexpectedly high mortality observed even among groups receiving the lowest doses of X radiation (85% at 650 r, 92% at 700 r, etc.). More striking, however, was the observation that the incidence

10. Finney, D. J. 1947. Probit analysis. Great Britain, Cambridge University Press.

TABLE 2.—*Mortality and incidence of infection in X-irradiated rats.*

Exp. no.	Series	Treatment	Mortality LD_{50}(r) (method of probits)	Incidence of infection*					
				Nonsurvivors		Survivors†		Total	
				Infected/total	Percent	Infected/total	Percent	Infected/total	Percent
1		Irradiated	<650	100/290	35	2/10	20	102/300	34
		Control				0/20	0	0/20	0
2		Irradiated	438 ± 18‡ (402–474)	21/69	30	35/90	39	56/159	35
		Control				66/163	40	66/163	40
3	1	Infected Irradiated	569 ± 22 (528–614)	21/54	39	2/43	5	23/97	24
	2	Infected Control				8/105	8	8/105	8
	3	New stock Infected Irradiated	625 ± 143 (399–982)	8/47	17	2/48	4	10/95	10
	4	New stock Infected Control				0/45	0	0/45	0
	5	New stock Irradiated	703 ± 16 (673–734)	6/37	16	0/38	0	6/75	8

* Doubtfully infected and/or cannibalized animals are omitted from infection data but are included in mortality data.
† Irradiated survivors were sacrificed at 30 days: groups of controls were sacrificed at 30 days, and in addition (exp. 2 and 3) at several earlier intervals.
‡ Standard error of LD_{50}: brackets indicate 95% confidence intervals.

of pulmonary lesions increased markedly with postirradiation time; for example, 33% of ten rats that died on the sixth day, 40% of 48 on the eighth day, 63% of 40 on the tenth day and 89% of nine on the 12th day. The total incidence of infection in the irradiated rats, of those that died and of survivors, all of which were sacrificed at 30 days, was 33%. This is to be compared with a weekly cumulative incidence of 5 to 10%, and biweekly incidence of about 34% in the colony as a whole. However, in the control group of 20 rats, treated in a manner identical with that of the experimental rats except for the irradiation, no infection was found at the end of the 30-day period.

It is apparent from these results that a synergistic relationship existed between X irradiation and the infection. Mortality was excessive at dose levels where little or no lethality was expected on the basis of previous experience in this laboratory. Since, in this experiment, deaths occurred in many rats not exhibiting lung lesions, it is likely that gross pulmonary pathology did not represent all manifestations of the disease.

A marked difference between the time distribution of infection in the irradiated rats that died and that of the nonirradiated controls was observed. The data suggested that a synergistic mechanism leading to death was operative before the appearance of grossly evident lesions. Probably many rats, particularly those with relatively short survival times, died as a result of the combined injuries before the infection became grossly detectable. Too few animals remained alive to permit a comparison of the incidence of infection in survivors and nonsurvivors in this experiment.

Experiment 2 yielded an X-ray LD_{50} of 438 ± 18 r for rats in the infected colony, a value considerably lower than was seen in normal animals at this laboratory. The incidence of pulmonary lesions was similar among the irradiated nonsurvivors and nonirradiated controls. An increase in incidence of infection with survival time was not as clear-cut as in experiment 1. Comparison of the several control groups sacrificed at intervals during the 30-day period revealed no definite trend toward either increase or decrease of the incidence of infection in the colony over this length of time.

Once again, in experiment 3 (series 1), rats from the infected colony exhibited an abnormally low X-ray LD_{50}, 569 ± 22 r. The LD_{50} value obtained with fresh Sprague-Dawley stock newly exposed to infection by being caged with infected animals (series 3) was 625 ± 143 r.* No significant difference in radiosensitivity could be demonstrated between this group and the others because of this large standard error. New Sprague-Dawley stock not deliberately exposed to infection (series 5) gave an LD_{50} of 703 ± 16 r, a much more plausible value for normal animals, although even in this group some infection appeared. In experiment 3 a definite trend was seen toward increased incidence of pulmo-

* This LD_{50} value was computed by the method of probits for the following data:

Dose in r	Dead/total
300	0/15
400	2/15
500	2/15
600	1/15
700	12/15
800	15/15

The large standard error observed can be attributed chiefly to the low mortality at 600 r. If the probit regression line is calculated from all of the above data, the probability that this point is derived from the same population as the remainder of the points is less than 1 in 1000. If the regression line is recalculated without this point, a value of 581 ± 25 r is obtained.

nary lesions in the irradiated infected population, as compared with the irradiated "normal" herd and with nonirradiated infected controls. In the irradiated females (series 1) total incidence of infection was 24%, as opposed to 8% in the nonirradiated controls (series 2). The previously noted effect of irradiation on the spread or "activation" of the disease was substantiated to some extent by the results in series 3, 4, and 5. In series, 3 the irradiated males intimately exposed to the infection developed an incidence of 10%, whereas no infection appeared in the nonirradiated controls (series 4). The rats in series 5, which were maintained in clean, separate quarters, and which were associated with the disease after arrival at this laboratory only through the medium of the caretaker, showed an incidence of infection of 8%. Whether the new stock was entirely free of pulmonary infection on arrival was not ascertained. Occasionally some incidence of pulmonary lesions has been observed in new rat stock supplied by outside sources to this and other laboratories.[11]

In the weekly screening of animals bred in the laboratory it was noted that the greatest incidence of infection occurred in rats seven to ten weeks of age. When very young animals (three weeks old) were removed to another laboratory free of infection, they showed a very low incidence of infection (5%) at autopsy three months later, while litter mates retained at this laboratory in rooms with known infected animals showed an incidence of 25%.

Both the number of infected lobes and the extent of involvement of each lobe were observed in many animals during the course of these studies. No significant alteration in the character or extent of the pulmonary lesions could be ascribed to irradiation. It was the definite impression of the authors that the extent of generalized hemorrhage seen in the infected animals that died after irradiation was considerably greater than that seen in the noninfected animals.

If the mortality data from all three experiments are pooled, the X-ray LD_{50} for rats with pulmonary lesions is 420 ± 50r, as opposed to 600 ± 45 r for rats from the infected colony found to be grossly normal. Neither the time to 50% death after irradiation nor the time of peak incidence of death was significantly different in the irradiated infected herd as opposed to the irradiated "normal" population.

The differences in the results obtained in the three experiments may be due to the fact that they were carried out over a period of several months. Apparently the extent and infectiousness of the disease changed considerably during this time. At the time the first experiment was done, the disease could be maintained at endemic proportions by the screening out (at weekly intervals) of animals obviously infected. However, if young animals (12 to 14 weeks old) were not culled for a month's time, the cumulative incidence of infection was 46% at the end of 30 days. At the time experiment 2 was run (three months after experiment 1), the incidence of disease had assumed epidemic proportions even among frequently screened animals, and it is possible that nearly all susceptible animals had contracted the disease by this time.

Since the incidence in the infected colony had dropped considerably by the time experiment 3 was run (8%, even among animals unscreened for one month), it might be postulated that a large number of animals had become immune. The lesser incidence of infec-

11. Finkel, M. P. (Argonne National Laboratory Chicago, Ill.) Personal communication.

tion in irradiated survivors from the infected colony as opposed to nonsurvivors (series 1) might support this contention. However, the fact that the new Sprague-Dawley rats showed a similar trend after irradiation (series 3) and, in addition, failed to develop infection even after intimate contact with infected animals (series 4), would suggest a change in the character of the disease.

SUMMARY

Advantage was taken of a naturally occurring endemic pulmonary infection in a colony of laboratory rats to study (a) the effects of irradiation on the incidence and severity of the natural infection in the colony, and (b) the degree to which the infection influenced X-ray morbidity and mortality. The data strongly suggest that irradiation increases the rate of spread of the disease or "activates" infection that otherwise might remain latent. However, these effects could not be evaluated accurately since the incidence of disease in nonirradiated rats varied considerably with the stage of development of the endemic infection. The X-ray LD_{50} for rats showing gross evidence of a disease that in itself did not kill immediately is reduced considerably below that for apparently normal animals.

These findings re-emphasized the necessity of maintaining a healthy experimental animal colony, particularly in investigations extending over several weeks and involving an agent, such as X ray, which not only results in general debility but also can interfere with specific immune mechanisms.

THE CULTIVATION OF THE EXOERYTHROCYTIC FORMS OF *PLASMODIUM GALLINACEUM* IN TISSUE CULTURE

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This report presents a series of investigations dealing with the cultivation of the exoerythrocytic forms of *Plasmodium gallinaceum* in tissue culture. These studies comprised two main lines of investigation: one, a survey of various tissues of infected chick embryos to determine their suitability as a source of explants for cultures, and two, the determination of the effects of various environmental factors on the parasites and the host cells. These studies were suggested by the work of Hawking (1945), who had a short time before accomplished the successful cultivation of exoerytherocytic forms of malarial parasites. At the time the present studies were begun the only published confirmation of Hawking's work was that of Zuckerman (1946). The purpose of the present studies was to extend the work of Hawking, especially with the view of studying the metabolism of the exoerythrocytic forms in vitro. After the present work was begun several other reports appeared along these lines, the most extensive being that of Lewert (1950). The literature dealing with the in vitro cultivation of exoerythrocytic forms of malarial parasites has been thoroughly reviewed by Porter (1948) and by Lewert (1950) and will not be summarized here.

Received for publication December 13, 1951.

This work, which was carried out at the University of Tennessee between 1947 and 1949, was made possible through cooperation of the Tennessee Valley Authority through financial assistance provided under contractual agreement, and through a contract with the Office of Naval Research, Microbiology Branch, Number N8onr-75700.

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METHODS AND MATERIALS

Culture flasks.—In preliminary studies the method employed by Hawking was used, which was essentially similar to the method suggested by Jacoby (1944), in which small coverslips are cemented with a plasma clot to the bottom of a Carrel flask. This method allows for removal of individual coverslips for study without disturbing the rest of the cultures. It was soon found, however, that the method used by Zuckerman (1945) was more suitable to the present work. This method, therefore, with a few modifications, was used exclusively for most of the present studies. The procedure consists essentially of growing cultures on coverslips which have been cemented onto bottomless Carrel-type flasks. This has all the advantages of a flask type of culture together with the advantage of obtaining permanent, stained preparations in which all the cells and parasites in a single flask culture can be examined microscopically. The details of the method, including a description of the flasks, are available in a paper by Zuckerman (1945) and in another by the present author (Dubin and Yen, 1950).

Early in the work cultures were made from various tissues of infected chick embryos by embedding the explants in a plasma clot consisting of a mixture of heparinized chicken plasma and chick embryo juice. In later studies, dealing with the effects of environmental factors on the parasites, the method consisted of the cultivation of macrophage cultures directly on the coverslip without the use of a plasma clot. Excellent cultures of macrophages are obtained by this procedure when splenic fragments are used as implants. The pieces of infected spleen are merely placed on the coverslip and covered with liquid nutrient medium. The macrophages rapidly migrate from the implants and become firmly attached to the coverslip as a single cell layer. There are several advantages to the use of such a system: (1) the omission of the plasma clot saves considerable time and effort, (2) the ab-

sence of the clot ensures a pure culture of macrophages in the great majority of instances, (3) the macrophages and parasites (both intracellular and extracellular) are exposed directly to the liquid medium without the intervention of a plasma clot, and (4) the single layer of cells on the coverslip allows for excellent staining and provides good cytological detail of cells and parasites.

Nutrient medium.—The nutrient medium consisted of 20% fresh chicken serum in Tyrode's solution (Parker, 1950), with phenol red as indicator in a final concentration of 0.0025 to 0.005%. The nutrient was changed every two or three days, depending upon the rate at which the pH diminished. Preliminary studies indicated that under the conditions of our experiments best results were obtained when the nutrient was changed every third day. No antibiotics were incorporated into the medium, except in the series of experiments dealing with the inoculation of sporozoites into cultures of normal macrophages. Hawking (1945) routinely used 3 to 5 units of penicillin per ml of nutrient medium.

Sources of tissue cultures.—Tissue cultures were set up from various tissues of chick embryos infected with the exoerythrocytic stages of *P. gallinaceum*. Ten-day-old chick embryos were infected by the chorioallantoic route (Zuckerman, 1946) with pieces of chick embryo brain containing numerous exoerythrocytic forms of the parasite. Six days later blood smears were examined for the presence of malarial parasites. The blood smears were obtained by making a slight tear in the shell membrane near the blood vessels, whereupon a slight oozing of blood occurred onto the chorioallantois. A sterile glass slide was then touched to the blood. Small numbers of erythrocytic parasites and exoerythrocytic stages within circulating monocytes were found in such blood smears within six to seven days after inoculation of the embryos. At such a time, usually within six to seven days after infection, cultures were set up from various tissues of the infected embryos. Smears of the brain and spleen were made at this time, stained with Wright's stain and examined for the presence of exoerythrocytic forms. Usually such embryos contained a rich supply of these forms, especially in the brain, liver and spleen.

Control of pH.—The pH was controlled in two ways. In those experiments where a constant pH was desired, control was secured by a continuous flow of gas mixtures containing oxygen, nitrogen and carbon dioxide. The details of this procedure are described elsewhere (Dubin and Yen, 1950). Otherwise, the cultures were gassed at the time of planting with either the carbon dioxide mixtures mentioned above or with expired alveolar air filtered through a sterile capillary pipette, one end of which was plugged with cotton. The flasks were then tightly sealed with rubber stoppers. As these cultures became more acid, the pH was adjusted by loosening the stoppers and allowing the accumulated carbon dioxide to escape; when the desired pH was reached, the flasks were again tightly stoppered.

Duration of cultures.—Throughout the present work short-term tissue culture studies were used, since it was expected that the various factors to be studied would exert their effects within a few days. Consequently, cultures were usually allowed to grow only five to seven days. This period of time allowed the exoerythrocytic forms to go through at least two cycles.

All cultures were grown at 37 to 38 C.

Fixing and staining.—In the early phases of the work the cultures were fixed with Zenker-formol and stained with Maximow's stain, according to the procedure used by Zuckerman (1945). This was found to be too time-consuming, however, and the following procedure was adopted. At the end of the experiment the nutrient was removed, the flask rinsed quickly with saline solution to remove residual serum proteins, and after the saline solution was poured off, about 1 ml of absolute methyl alcohol was added to the flask. This served to wet-fix the culture. The flask was then set in a large Stender dish containing absolute methyl alcohol, to dissolve the cement.* Usually the cement was dissolved within two hours. The implants in the case of the plasma clot cultures were then gently removed from the surrounding clot. The coverslips were then rinsed for three minutes each in three or four changes of absolute methyl alcohol to remove the remainder of the cement. The coverslips were dried in air and stained for 45 minutes with 2% Giemsa in distilled water buffered at pH 7.0. The stained coverslips were rinsed in water, drained and dried in air. When thoroughly dry they were mounted in clarite on a 3×1⅛ inch slide for permanent record.

Estimation of measurements of growth.—The results were evaluated for (1) the amount of cellular growth, (2) the cell types present in the outgrowths, (3) cytological differences in the cells between test and control groups, and (4) presence of malarial parasites. In the case of the macrophage cultures, the amount of growth of macrophages was estimated in terms of the area

* Gelva V-7, made by Shawinigan Products Company, New York City.

of the coverslip covered by the macrophage cultures. The growth of the parasites was estimated by the ratio of parasites to host cells or by number of parasites per unit area of coverslip.

EXPERIMENTAL RESULTS

1. Results obtained from different tissues

Cultures were prepared from spleen, brain, liver, lung, bone marrow, skeletal muscle and myocardium. Exoerythrocytic forms were found in cultures prepared from all of these tissues, although heavily parasitized cultures were found with regularity only in those derived from the spleen, liver and brain.

Certain generalizations concerning the cellular activity in the cultures are pertinent at this point.

(a) *Cultures in plasma clot.*—In the plasma clot cultures there were three distinct areas of activity—namely, (1) the inner zone, immediately beneath the implant, (2) the middle zone, in the area of the clot itself, and (3) the outer zone, the area of coverslip beyond the limits of the plasma clot. In general, the predominant cell type in the inner zone was the macrophage, and that of the middle zone was the fibroblast or endothelial cell or both, depending upon the nature of the implant. Where the outer zone contained any cells at all the predominant cell type was also the macrophage. Other special elements, such as epithelial or nervous tissue cells, were found primarily in the inner zone.

(b) *Macrophage cultures grown without plasma clot.*—In macrophage cultures derived from the spleen in which no plasma clot was used, two areas of activity were seen in each colony of a coverslip culture—namely, the inner and outer zones. The area of the inner zone of a colony consisted of the site immediately beneath the splenic fragment. Between the piece of spleen and the coverslip a colony of macrophages developed which was temporarily covered by the implant. After the fragment was moved by tilting the flask, a new colony was started at the new site at which the piece of spleen came to rest. In addition to this, there was outgrowth of macrophages beyond the central area into the outer zone. When conditions were generally unfavorable to cells and parasites, the greatest regressive changes occurred in the outer zone, while lesser changes were seen in the inner zone.

In addition to these general results, there were special findings which varied with the histological nature of the implant. These will be discussed below.

Spleen.—(a) *Plasma clot cultures.*—In those cultures in which the splenic fragments were embedded in plasma clot, there were two predominant cell types, the macrophage and the fibroblast. In the inner zone there was a marked preponderance of macrophages, although fibroblasts were also present in small numbers. An outgrowth of fibroblasts was present in the area of plasma clot. Macrophages were also seen in this middle zone, but they were generally few in number and presented a cytological appearance quite different from those seen in the inner and outer zones. The former were more elongated or astrocytic and had several large vacuoles and considerably less cytoplasm. Beyond the limits of the plasma clot the outer zone contained numerous macrophages which once again resembled the macrophages present in the inner zone. One gained the impression that the plasma clot was generally inhibitory to the macrophages. Indeed, as will be shown later, this inhibitory effect was at times quite striking in its influence on the other cell types as well.

Exoerythrocytic forms of the parasites were present in both macrophages and fibroblasts but were much more numerous in the former. Also, the para-

sites tended to be more numerous in the inner zone than in the other areas. Nevertheless, parasites within macrophages were found within the outer zone as well. Although parasites were found within fibroblasts, they tended to be concentrated in the inner aspects of the middle zone, immediately adjacent to the implant, and very few were seen in the outer margins of the area of fibroblastic outgrowth.

(b) *Coverslip cultures without plasma clot.*—Practically a pure culture of macrophages developed in those cultures in which splenic fragments were not cemented to the coverslip with plasma clot. Splenic granulocytes and lymphocytes also wandered out from the implant, but these cells generally disappeared within a few days, the granulocytes first. If the splenic fragments were allowed to remain in one position for a matter of 24 hours or more, they sometimes became fixed to the coverslip by a sparse outgrowth of fibroblasts. This could be avoided by tilting the flask once or twice daily.

Excellent cultures of macrophages were obtained by this method, and if as many as three pieces of splenic fragments were introduced into a flask, the coverslip would be almost completely covered by macrophages within three or four days. Where the growth of macrophages was less rich, the two zones (inner and outer) were distinctly visible in each colony within a flask. Exoerythrocytic forms in large numbers were found in such cultures. While these tended to be more concentrated in the inner zones, they were found in all parts of the coverslip, and the concentration within the inner zone was not as marked as in the plasma clot cultures.

All stages of development of the exoerythrocytic forms of the parasite were seen in the cultures—namely, very young round forms, small and large schizonts, mature segmenters, rupturing segmenters, and liberated merozoites either lying free in the extracellular environment or engulfed by new host cells. The only forms seen extracellularly in the cultures were merozoites.

The parasites appeared to have no damaging effect upon the host cells. This applied to the macrophage and the fibroblast, as well as to the endothelial cell which grew out from the pia mater of the brain implants, to be described below. The only evidence of damage seen was in those macrophages in which segmenters had ruptured. In this case there appeared to be a lysis or destruction of the cytoplasmic membrane, but even so the nucleus appeared relatively normal. Whether such cells died or recovered was not determined. In any case, there was no cytological evidence of the presence of a diffusible toxin which was capable of injuring neighboring uninfected cells. Moreover, the macrophage is a very sensitive cell which quickly reflects unfavorable conditions in the surrounding medium, and such a cell would have presented definite cytological evidence of damage had the intracellular growth of the parasite seriously interfered with the cellular metabolism.

The results obtained with the plasma clot cultures of the remaining tissues were much more variable than those obtained with the splenic cultures. The inner zones always contained good numbers of macrophages, but the cellular outgrowths into the plasma clot and beyond varied considerably, for reasons which were not understood. It seemed that at times the plasma clot inhibited the migration of macrophages as well as the outgrowth of fibroblasts and endothelial cells. This occurred frequently enough so that if it became necessary

to work with cultures of fibroblasts or endothelial cells consideration would have been given to the utilization of some other type of physical substrate, such as cellophane (Evans and Earle, 1947).

Liver.—In the inner zone, there was a moderately good growth of macrophages, which contained numerous exoerythrocytic forms in all stages of development. At times there were also outgrowths of small islands of epithelium which resembled liver parenchyma. At no time, however, were parasites found within the liver epithelial cells, even though heavily infected macrophages were lying immediately adjacent. This is interesting in the light of the finding of pre-erythrocytic forms of mammalian malarial parasites within the liver cell and nowhere else (Shortt et al, 1949). Within the plasma clot there were outgrowths of fibroblasts, some of which contained exoerythrocytic forms. At such times when macrophages were found within the middle and outer zones they also contained exoerythrocytic stages of the parasites.

Brain.—The brain fragments were deliberately selected to contain some overlying pia mater. Within the inner zones there were large numbers of macrophages which were usually heavily infected with exoerythrocytic forms. Occasionally this zone also contained some cells which appeared to be of nervous origin. These consisted of small islands of small, tightly packed cells which contained small, dark, round nuclei with negligible amounts of cytoplasm. Some of these islands appeared to consist of neuroepithelium and others of type B Schwann cells. Out of these islands grew slender, elongated nodose structures which consisted of several nuclei strung out in series and connected by a delicate filament. These resembled type A Schwann cells (Murray and Stout, 1940 and 1947). No parasites were found within these nervous elements. In the inner zone there were occasionally also present small sheets of unidentified cells which were somewhat suggestive of endothelial cells but which, unlike the identifiable endothelial cells, were always free of parasites.

Within the plasma clot outgrowths of fibroblasts were present, as well as smaller, more slender, elongated cells which were taken to be endothelial cells. Many exoerythrocytic forms were found within these cells, especially at the inner aspect of the clot, close to the implant. More parasites were found within the endothelial cells than within the fibroblasts. In some cultures small numbers of macrophages penetrated into the plasma clot and beyond.

Lung.—As a rule, the number of parasites in the lung cultures was small compared to the number in the cultures derived from the spleen, liver and brain, but occasionally the exoerythrocytic forms in such cultures were fairly numerous.

Within the inner zone there was usually a good outgrowth of macrophages which contained exoerythrocytic forms in all stages of development. In addition, this zone also contained sheets of cells which suggested epithelium. These epithelial sheets sometimes reached a comparatively large size. Small numbers of exoerythrocytic forms were found within these epithelial-like cells. It is interesting to observe that such forms were always either very young, round forms with a single chromatin dot or small, immature schizonts. No large schizonts or mature segmenters were ever seen within these epithelial-like cells. Indeed, at times these cells contained large oval intracytoplasmic inclusions which sug-

gested "ghost" forms of dead schizonts. Such inclusions were not seen in other cell types. This suggested the possibility that the exoerythrocytic forms were able to develop in these epithelial cells only up to a certain point and then died out. This brought to mind the limited development of pre-erythrocytic stages of various avian malarial parasites in hosts with partial natural immunity (Huff, 1951).

Within the plasma clot there were outgrowths of fibroblasts which contained small numbers of exoerythrocytic forms.

Bone marrow.—The predominant cell types in the bone marrow cultures were macrophages and fibroblasts. Both of these contained exoerythrocytic forms in small numbers. The bone marrow, however, was cultivated a few times only, so this paucity of exoerythrocytic stages in these few instances may not be characteristic of cultures from this source. Hawking (1945) obtained good results with cultures from bone marrow.

Heart and skeletal muscle.—Results obtained from the heart and leg muscles were similar, producing good outgrowths of fibroblasts, with only small numbers of macrophages. Very few exoerythrocytic forms were seen and these were found in both types of cells. The origin of these exoerythrocytic forms in the cultures of heart and skeletal muscle was not clear. They may have come from infected circulating monocytes within capillary vessels included in the implants or from infected fibroblasts or capillary endothelium.

2. *Effects of various environmental factors*

The macrophage cultures derived from infected chick embryo spleen and grown without plasma clot clearly provided the best system for the study of effects of environmental factors on exoerythrocytic stages in tissue culture. Moreover, the exoerythrocytic forms were generally more numerous in such cultures than in those prepared from other tissues. This method, therefore, became the method of choice for all subsequent studies, with a few exceptions. It is understood that this method was used in all work reported unless otherwise indicated.

The chief disadvantage of using the chick embryo spleen was its small size. This difficulty was surmounted by using several spleens in a single experiment. The details of the design of individual experiments have been described in a previous report (Dubin and Yen, 1950).

Heparin.—It was decided to test the effect of heparin because some workers have suggested that heparin is inhibitory to cells in vitro. In this series of experiments splenic fragments were embedded in a clot prepared by mixing chick embryo extract and chicken plasma. The embryo extract was common to both control and test groups. In the control group, plasma was used which had been obtained without the use of an anticoagulant. This was done by drawing heart's blood with a syringe coated with mineral oil and preparing the plasma in paraffined tubes. The heparinized plasma was prepared by using 1.5 ml of a 1:1000 solution of heparin for 15 ml of blood.* Assuming that all the heparin remained in the plasma component of the blood, the final concentration of heparin in the plasma clot was approximately 1:7000. No differences were observed between the control and test groups, either as to amount and appearance of cellular outgrowths or relative numbers of exoerythrocytic forms.

At one time the only available heparin

* This solution was prepared from powdered heparin obtained from the Connaught Laboratories, Toronto, Canada. The powder contained 110 units per mg.

was in the form of a solution which contained phenol as a preservative. When this was compared with another batch of heparin solution which contained no phenol, no differences were noted in the cultures. In this experiment, the final concentration of phenol in the plasma was approximately 1:4000.

Heterologous medium.—The effect of heterologous serum proteins was tested on plasma clot cultures of spleen, liver and brain removed from infected chick embryos. Chick embryo extract was used as the coagulant. Three experimental groups were set up. In the control group (group 1) the clot was prepared from chicken plasma and the overlying liquid nutrient medium consisted of dilute chicken serum. In group 2 the nutrient medium was the same, but the clot was prepared with human plasma. In group 3 both the plasma clot and the dilute serum were of human origin. There were no differences between groups 1 and 2, but in group 3 there was a complete suppression of macrophage growth, the only cellular outgrowth consisting of a sparse collection of fibroblasts. This fibroblastic outgrowth was less than that in the other groups. Rare exoerythrocytic forms were still present, however, in the fibroblasts grown in the completely heterologous medium.

Lewert (1950) also noted poor results when chick macrophage cultures were grown in the presence of a heterologous medium consisting of beef-embryo extract and human placental serum. This he interpreted as being due primarily to the sensitivity of macrophages to tissue extract (Baker, 1933). This explanation seems unlikely, since considerably higher concentrations of tissue extract are needed to seriously affect the macrophage cultures, as will be seen later in this report. Moreover, in the present experiments, the deleterious effect on the macrophages was clearly related to the presence of the heterologous serum.

Hydrogen ion concentration.—Since there was considerable shift of pH in the stoppered culture flasks during the period of cultivation, it seemed desirable to learn the range of hydrogen ion concentration over which optimal growth of the parasites occurred. Indeed, there was no information concerning the pH tolerance of the host cell itself, the macrophage. Accordingly, a series of experiments were set up to determine the complete range of pH tolerated by macrophages and exoerythrocytic forms when grown in vitro at constant pH levels for periods of days. These experiments are described in detail in a previous report (Dubin and Yen, 1950).

Optimal growth of macrophages occurred between pH 6.8 and 8.2. Lesser degrees of growth occurred at pH 6.6 and 8.4 and ceased entirely at pH 6.4 and 9.0. The exoerythrocytic forms displayed a narrower pH tolerance than the macrophages. They grew equally well between pH 7.2 and 8.0. At pH 7.0 growth fell to about 50% of the control and at pH 6.8 to about 25%. No parasites were seen at pH 6.6. At pH 8.2 growth decreased to about 50% and at pH 8.4 to about 25%. Rarely were parasites seen up to pH 9.0.

Interesting morphological differences were noted in the appearance of the macrophages at the extremes of the pH spectrum. These are discussed in the previous paper.

Indicators.—During preliminary work with the effect of pH on the cultures, phenol red, cresol red and metacresol purple were tested in final concentrations of 0.005%. Equally good growth of macrophages and parasites occurred in all groups.

Osmotic pressure.—Since it was planned to vary the culture medium in

future experiments, information was required concerning the range of osmotic pressure tolerated by the macrophage and the malarial parasite. As in the case of the pH tolerance, no such information was available in the literature. A series of experiments was set up to determine the effect of various levels of osmotic pressure on macrophages and parasites. The experiments are presented in detail elsewhere (Dubin, 1950). Optimal growth of both macrophages and exoerythrocytic forms occurred between 7 and 11 atmospheres of pressure. The growth of macrophages was reduced to 50% at 5 and 13 atmospheres and ceased at 4 and 15 atmospheres. Growth of the parasites fell to 50% at 6 and 12 atmospheres and ceased at 5 and 14 atmospheres.

Concentration of serum.—During the experiments dealing with osmotic pressure, it was noted that high concentrations of serum were inhibitory to both the malarial parasite and the macrophage. This phenomenon was studied in subsequent experiments, and it was found that the inhibitory effect varied from one batch of serum to another. Generally, concentrations of serum over 50% showed this inhibitory effect. Moreover, the exoerythrocytic forms were more sensitive than the macrophages to this harmful effect. In some experiments, for example, the presence of 50% serum resulted in smaller, more darkly-staining macrophages than in the control (20% serum), although the number of cells was not reduced; in the same cultures, however, there was a definite drop in the number of parasites in the test group which contained 50% serum. At such times when no inhibitory effects were produced by a serum concentration of 50%, diluting the serum from 50 to 20% produced no obvious differences. Consequently, all future experiments, with certain exceptions to be noted below, were carried out with 20% serum.

The inhibitory substances in the serum were not identified. All serums were obtained from young birds by cardiac puncture under sterile conditions and were never filtered. Chemical contamination from the glassware was considered but seemed unlikely.

Hawking (1945) noted that growth of his cultures was not much more vigorous if the proportion of serum was increased to 50%. He therefore chose 20% as an economical level.

Lewert (1950) noted that when the proportion of chicken serum in the supernatant medium was reduced from 4 parts in 6 to 1 part in 6, there was no marked effect on the rate of growth of his cultures.

"Lipoprotein" serum precipitate.—It was noted that chicken serum, which was perfectly clear at the time of the original preparation, developed a flocculent sediment on standing in the refrigerator. These batches were repeatedly cultured for bacteria but always with negative results. It was suggested by several biochemists that this sediment probably consisted of some form of lipoprotein. No chemical tests were performed on this sediment.

Several experiments were done to see whether this sediment might have either a harmful or beneficial effect on the cultures. In one typical experiment the sediment was removed by centrifugation from a batch of chicken serum which was two weeks old. The sediment was suspended in Tyrode's solution and was used as the sole nutrient in one test group (group 1). In group 2 the nutrient medium consisted of the clear supernatant from the same batch of serum diluted with Tyrode's solution. In group 3, dilute fresh chicken serum

was used. No differences were noted between groups 2 and 3. The cultures in group 1, however, grew very poorly; it appeared that the materials in the sediment were not capable alone of maintaining the growth of the cells or parasites.

In another typical experiment the effect of a higher concentration of this sediment was tested. Beginning with 40 ml of a batch of pooled serum, the sediment was centrifuged down, and 20 ml of clear supernatant serum were removed to serve as nutrient for one test group. The sediment was then resuspended in the remaining 20 ml of serum, thereby doubling the concentration of sediment originally present in the serum. This served as nutrient for the second group of cultures. Again, no differences were noted in the two test groups.

In summary, no harmful or beneficial effects could be traced to the presence of this sediment in the nutrient medium.

Chick embryo juice.—The effects of chick embryo juice were tested because, although Hawking had included it in his nutrient medium, embryo juice was known to have a damaging effect on macrophages (Baker, 1933). Hawking found that although embryo extract was not essential, its presence caused more rapid growth. The concentration of embryo extract in his nutrient medium was roughly equivalent to about 2% of pure chick embryo juice.

In the present experiments the embryo juice was prepared from 10-or 11-day-old chick embryos by handchopping the embryos and spinning down the tissue fragments. The clear supernate was designated as 100% chick embryo juice.

In one experiment splenic fragments were set up in a medium consisting only of chick embryo juice diluted with Tyrode's solution. In this experiment the concentration of embryo juice varied from 1 to 20%. There was no growth at all in these flask cultures.

In another experiment varying concentrations of embryo juice were added to the nutrient medium, which consisted of 10% chicken serum in Tyrode's solution. One percent embryo juice had no effect either on the macrophages or on the parasites. Ten percent embryo juice, however, produced slight cytological changes in the macrophages and a definite reduction in the number of parasites. With the use of 20% embryo juice the number of parasites was markedly reduced and the damage to the macrophages was increased. In this latter group the macrophages were considerably smaller, contained much less cytoplasm, were rounded in shape and stained dark blue with Giemsa.

These results are consistent with those obtained by other workers in studies on the effects of embryo extract on macrophages (Carrel and Ebeling, 1926; Baker, 1933).

Glucose.—In the following set of experiments Krebs-Ringer-bicarbonate solution was used as diluent for the serum. This solution, which consists of a series of solutions each of which is isotonic, allows for variation in the concentration of individual ingredients without affecting the final osmotic pressure (Umbreit et al, 1948). This solution will be referred to as KRB. Desired amounts of glucose were added to this solution from a solution of glucose in isotonic sodium chloride solution. The amount of sodium chloride solution in KRB was correspondingly decreased in order not to affect the final concentration of other substances in the medium. A batch of pooled fresh serum was obtained from starved chickens and was measured for glucose content. Twenty

percent of this serum was used in all experimental groups. The concentration of glucose in the different test groups varied from 50 mg% to 1100 mg%. The pH was kept constant by continuous flow of a 2% carbon dioxide gas mixture. The cultures were examined at the end of four days and showed equally good growth of cells and parasites in all experimental groups.

Calcium, inorganic phosphorus and bicarbonate.—In preliminary studies on the effect of pH on the cutures, both Krebs-Ringer-bicarbonate (KRB) and Krebs-Ringer-phosphate (KRP) solutions were used as diluents for the serum. Much better results were obtained with KRB than with KRP. KRP, in spite of its high phosphate content, had a much poorer buffering capacity than KRB. Moreover, the growth of cells and parasites was poorer in KRP, and the cells in the outer zones of the cultures were shrunken. This was associated with a precipitate of some form of calcium phosphate. Several possible explanations for this harmful effect on the cultures were considered. These were: (1) the precipitate itself, (2) the high level of inorganic phosphorus, (3) the low level of calcium, calcium having been made unavailable both by partial deionization and precipitation at pH 7.4, and (4) the low level of bicarbonate, since in a mixture consisting of 20% serum in KRP the only bicarbonate present was that contributed by the serum. Consequently, experiments were set up to study this question and, at the same time, to determine the effects of various concentrations of calcium and phosphorus in the nutrient medium.

Levels of calcium and phosphorus in the batches of pooled chicken serum were determined by standard chemical procedures. The KRB and KRP solutions were modified by the addition of glucose in isotonic sodium chloride solution to give a final concentration of glucose of 1:1000. The calcium and phosphate solutions in KRB and KRP were varied to give the desired final concentrations of calcium and phosphorus in the dilute serum mixture. Generally, KRB was used as the basic solution, and the phosphate ingredient solution of KRP was added to it in varying amounts. Because of the effects of pH on ionization and precipitation of calcium phosphate, the pH was kept constant at pH 7.4 by continuous flow of carbon dioxide gas mixtures.

By varying the proportions of calcium and phosphate solutions in the Krebs-Ringer mixture while keeping the pH constant, it was possible to achieve wide ranges of concentrations of calcium and inorganic phosphorus in the nutrient medium without the formation of a precipitate. To achieve low levels of these substances, the serum was reduced to 10% and controls run for this dilution of serum.

As a result of these experiments it was found that the macrophages and exoerythrocytic forms grew equally well in concentrations of calcium varying from 11 to 1070 mg per liter. Also, optimal growth occurred when the concentration of inorganic phosphorus was varied from 7 to 650 mg per liter. At 650 mg per liter the osmotic pressure was about 10 atmospheres, but this level is well within the limits for optimal growth, as was shown in earlier experiments. Then, by working within these limits of calcium and phosphorus levels and by empirically adjusting the relative proportions of the calcium and phosphate solutions in the KRB-glucose diluent, it was possible to obtain precipitates of calcium phosphate and show that the toxic effect was due to the precipitate itself. It was shown repeatedly that the increase of precipitate was associated with increasing damage to the

cultures, both cells and parasites being affected.

The effect of decreasing the bicarbonate in the nutrient medium was also studied. The bicarbonate content was decreased in the presence of both normal and reduced levels of inorganic phosphorus. The simultaneous reduction of bicarbonate and phosphorus was accomplished by using a low concentration of serum (10%) in Krebs-Ringer solution from which both bicarbonate and phosphate solutions were omitted. In this mixture all the bicarbonate and inorganic phosphorus were derived from the serum component of the nutrient. No measurements of the bicarbonate were made, the quantities being designated in terms of fractions of the amount present in normal serum.

In these experiments, where the buffers were so markedly reduced, the pH could not be kept constant, but adjustments of the pH were made with sodium hydroxide and hydrochloric acid solutions, to at least keep the pH within the optimum range for the host cell, namely pH 6.8 to 8.2. Consequently, while the following results are valid for the macrophage, one would be less certain concerning the interpretation of the results with respect to the parasites. When the bicarbonate was reduced to one-fifth the amount present in normal serum (in the presence of a normal level of phosphorus), there was a good growth of macrophages, but the parasites were slightly reduced in numbers. When both the bicarbonate and phosphorus were simultaneously reduced to one-fifth the normal value, the number of macrophages remained unchanged, but the cells were markedly shrunken, displaying a single large vacuole occupying the greater part of the scanty cytoplasm. The number of parasites was considerably reduced in this test group. When the bicarbonate was decreased to one-tenth of normal but in the presence of a normal level of phosphorus, the cells were somewhat shrunken although they were not reduced in number. The number of parasites, however, was reduced in this group. The most marked effects were seen in the group in which the bicarbonate and phosphorus were simultaneously reduced to one-tenth the normal level. In this group there was a marked reduction in the number of cells, and those that were present were extremely shrunken, the shrinkage affecting both nucleus and cytoplasm. Parasites were completely absent in this group.

In the light of earlier experiments it was clear that these effects on the macrophages could not have been caused either by the pH levels or by the decrease of inorganic phosphorus by itself. Apparently the cells and parasites were damaged by the decrease in bicarbonate concentration alone and were seriously damaged by the simultaneous decrease in the levels of bicarbonate and inorganic phosphorus.

Synthetic mediums.—Attempts were also made to cultivate tissues in synthetic mediums. The advantages of such a system for metabolic studies in vitro are obvious. Two such mediums were tried. One was the synthetic medium which had been utilized to study the growth in vitro of the erythrocytic parasite, *Plasmodium knowlesi* (Anfinsen et al, 1946). The other was the medium devised by White (1946). The latter was prepared both according to the original formula and according to a later formula in which additional amino acids were included (personal communication). Results were quite discouraging, the outgrowths being so scanty as to make further experimentation at that point unwarranted.

Jacoby and Darke (1948) tried the use of White's medium on pure cultures

of macrophages. They concluded that this medium had no toxic effect but that when used alone it proved insufficient to maintain the life and activity of pure populations of macrophages.

Attempts to infect cultures of normal macrophages with "cell-free" suspensions of exoerythrocytic forms or with fragments of infected tissue.—Attempts were made to infect cultures of normal macrophages with "cell-free" suspensions of exoerythrocytic forms. The method used was suggested by Dr. Harald N. Johnson of the Rockefeller Foundation (personal communication). Chick embryo livers, heavily infected with exoerythrocytic forms, were ground in 2% chicken serum in Tyrode's solution. This suspension was centrifuged at 800 r.p.m. for 5 minutes. This resulted in sediment I. The supernate was then spun at 2500 r.p.m. for 15 minutes, resulting in sediment II, which contained the exoerythrocytic forms, presumably cell-free.

When sediment II was inoculated by itself into culture flasks containing only standard nutrient medium, no cellular outgrowth of any kind developed. Sediment I by itself produced colonies of cells, chiefly fibroblasts. Some of these cells contained parasites.

When sediment I was inoculated into normal macrophage cultures a marked infection of these cultures resulted, the cultures showing numerous exoerythrocytic forms. Sediment II, however, gave poor results when inoculated into normal macrophage cultures, only very few of the cells becoming infected.

Another method used was the addition of pieces of infected brain or liver to the flasks in which either infected or normal splenic fragments had been placed. When such infected liver or brain (either in the form of tissue fragments or as a finely ground suspension) was added to the flasks already containing infected splenic fragments, there was a much better growth of macrophages than in the controls. This growth was better than could be accounted for by a simple summation of outgrowths from the spleen alone plus that from either the liver or brain. The impression was gained that the pieces of liver or brain acted as growth stimulants to the macrophages. The number of parasites, however, did not appear to be increased under these conditions.

When the pieces of infected brain or liver were placed in flasks simultaneously with normal splenic fragments, there was again an improvement in the macrophage cultures, but apparently no increase in the number of parasitized macrophages over and above that which might be explained by the simple outwandering of such cells from the infected brain or liver alone.

No attempts were made to inoculate cultures of normal macrophages with scrapings from infected tissue cultures.

Inoculation of sporozoites into cultures of normal macrophages.—Several investigators had attempted without success to infect cultures of normal cells with sporozoites of malarial parasites. This was accomplished for the first time in the present series of experiments by the present author working in collaboration with Dr. R. L. Laird and Mrs. V. P. Drinnon. The details of these experiments are reported elsewhere (Dubin, Laird and Drinnon, 1949 and 1950). In summary, the sporozoites of *P. gallinaneum* developed into cryptozoites in vitro, following the inoculation of salivary glands of infected *Aëdes aegypti* mosquitoes into cultures of normal chicken macrophages. Penicillin and streptomycin were added to the cultures to combat the growth of bacteria and fungi introduced with the salivary glands. A schedule of antibiotic dosage was worked out which allowed the maintenance of

bacteria-free cultures of pre-erythrocytic stages up to seven days of incubation. The complete development of cryptozoite from sporozoite occurred in about 48 hours. All stages of development of pre-erythrocytic forms were seen in the cultures, from young cryptozoites to merozoites which had been liberated by ruptured segmenters. By the end of seven days the young metacryptozoites seen in the cultures were considered to represent the fourth generation of pre-erythrocytic stages in the cultures.

Scrapings from these cultures were infective for chicks and produced a normal life cycle pattern (Laird, Dubin and Drinnon, 1950). The chicks developed erythrocytic forms, gametocytes included, and mosquitoes which were fed on such birds became infected. Sporozoites from some of these mosquitoes were in turn inoculated into chicks and into normal macrophage cultures and proved infective for both.

Antibiotics.—In the series of experiments dealing with the inoculation of sporozoites into normal macrophage cultures, penicillin and streptomycin had to be used to combat bacterial infection which resulted from the inoculation of the salivary glands. At that time we were not aware of the method developed by Gordon and Hill (1946) for obtaining bacteria-free suspensions of sporozoites from the salivary glands of infected mosquitoes.

Accordingly, the effects of penicillin and streptomycin, alone and in combination, were studied on the cultures of macrophages infected with exoerythrocytic forms. Penicillin G (Squibb) when used alone had no harmful effect on the host cells or parasites up to 5000 units per ml; higher concentrations were not studied. Dihydrostreptomycin (Squibb) was studied in concentrations up to 5000 μg per ml. At 400 μg per ml the cells were not affected, but there was perhaps a slight reduction in the number of exoerythrocytic forms when compared with controls. At 1000 μg per ml there was a moderate degree of damage to cells and parasites, while at 5000 μg per ml this damage was marked.

When penicillin and streptomycin were used in combination these antibiotics exerted a synergistic damaging effect on both macrophages and exoerythrocytic forms. The details of these studies are reported in a previous paper (Dubin, Laird and Drinnon, 1950).

DISCUSSION

In the present experiments exoerythrocytic forms of *P. gallinaceum* were cultivated in tissue cultures of several types of tissue. Parasites were most numerous in cultures of brain, liver and spleen, presumably because these tissues were most heavily infected initially. Hawking previously obtained good results with cultures of spleen, bone marrow, buffy coat of blood, and liver. The method employed by Hawking was conducive to the growth of both macrophages and fibroblasts, and exoerythrocytic stages were found in both these cell types. In his experiments no parasites were seen in cultures from brain, lung and heart. In our experiments numerous parasites were seen in cultures of brain, a moderate number in some cultures prepared from lung, and very few in cultures from heart and skeletal muscle.

Lewert's methods were primarily planned for the cultivation of fibroblasts and endothelial cells from pia mater and heart, although some work with macrophage cultures was also undertaken. Lewert observed parasites in all three types of cells. In general, it appears that Lewert's cultures contained relatively few parasites; furthermore, Lewert had difficulty in observing the parasites

microscopically. These results may have been caused by a combination of the following factors in his experiments: (1) cultures of fibroblasts, in the experience of the present author, contain much fewer parasites than cultures of macrophages or endothelial cells, (2) endothelial cells and fibroblasts growing in plasma clot tend to heap up in several layers, thereby obscuring visibility, and (3) the infected post-hatched chick (used as the source of explants by Lewert) contains considerably fewer exoerythrocytic forms than the infected chick embryo (Zuckerman, 1946).

(1) Concerning the first of these factors, the work of the present author has indicated that the fibroblast is the least favorable of the three types of host cells observed in the cultures. The exoerythrocytic forms were found in decreasing order of frequency in the following cells: the macrophage, the endothelial cell and the fibroblast. The cause of this difference was not clear. It was not determined in the present work whether the diminishing order of frequency in macrophage, endothelial cell and fibroblast is a direct reflection of a diminishing metabolic preference for such cells on the part of the parasite, or whether certain physical factors were responsible for this result. For example, the active motility of macrophages may afford a better opportunity for infecting other macrophages in a culture. One had the impression that, while such a factor was responsible for the greater frequency of infected cells at the periphery of macrophage cultures as compared with the ratio of infected cells in the same zone in cultures of fibroblasts or endothelial cells, still such a factor probably played a small role in the greater concentration of exoerythrocytic forms in macrophages rather than in the other two cell types. For even in macrophage cultures the greatest concentration of parasites always occurred immediately beneath and around the implant. It is the impression of the present author that the larger number of parasites in macrophages as compared with other cell types is best explained on a metabolic preference of the exoerythrocytic stages for the macrophage.

(2) The macrophages tend to spread out as a single layer of cells, while the endothelial cells and fibroblasts tend to heap up in the plasma clot. This, together with the density of the clot itself, makes for poor visibility at the very point where the exoerythrocytic stages are present in greatest number in plasma clot cultures, namely at the point in the clot immediately adjacent to the implant. In contrast, the macrophage cultures grown in the present experiments presented a single cell layer which allowed for excellent visibility, staining qualities, and cytological detail.

It would appear, therefore, that the methods described in the present paper would be the ones of choice for future studies on the effects of environmental factors on exoerythrocytic stages in vitro. There is the additional advantage that the cells grown in the absence of plasma clot are exposed directly to the nutrient medium without the intervention of plasma clot.

These methods could also be used for the study of the entire general problem of the relationship of exoerythrocytic to erythrocytic forms, and in particular, the development of the parasite at certain crucial points in its life cycle. These include development from sporozoite to cryptozoite, from pre-erythrocytic stage to erythrocytic stage, from erythrocytic merozoite to phanerozoite, and from phanerozoite back to erythrocytic trophozoite.

Hawking (1945) had carried out some

studies on the effects of environmental factors on the exoerythrocytic stages in vitro. These were relatively few in number, however. Lewert demonstrated alterations in the cycle of *P. gallinaceum* following passage of the exoerythrocytic forms in tissue culture. Tonkin (1946) and Zuckerman (1946) tested the effects of various drugs on the exoerythrocytic forms in tissue culture. These studies, together with the present ones, represent the only studies to date on the metabolism of the exoerythrocytic forms in vitro. The methods described in the present experiments and the data presented therein await further exploration along these lines.

The natural development of exoerythrocytic forms of *P. gallinaceum* takes place in certain mesenchymatous elements, namely, the macrophage, endothelial cell and fibroblast. It was interesting to note, therefore, that well-stained exoerythrocytic stages were observed within epithelial cells growing out from pulmonary implants. Apparently these forms were able to develop only incompletely within such cells, for no stages older than young schizonts were seen within the epithelium. The incompleteness of this development should be checked, of course, to make certain it was not the result of a chance finding. If confirmed, it would be analogous to the limited development of pre-erythrocytic stages of avian malarial parasites in hosts with partial natural immunity.

Lewert demonstrated that following the passage of exoerythrocytic stages of *P. gallinaceum* in tissue culture, these parasites produced an infection in birds which was almost exclusively exoerythrocytic in type. Hawking had reported earlier that the infection of birds from his cultures was slower than normal in developing erythrocytic stages of the infection. The results of our experiments (Laird, Dubin and Drinnon, 1950) were interesting in this connection. When chicks were inoculated with pre-erythrocytic stages grown in vitro (following development from sporozoites in tissue culture), the majority of such chicks survived, in spite of the fact that many showed parasitization of over 50% of the erythrocytes. This is in sharp contrast to the well-known observation that chicks infected with sporozoites usually die in from 14 to 20 days post-inoculation. Such differences may have been due to some alteration of the pre-erythrocytic stages in vitro. In contrast to Lewert's results, however, we obtained heavy infections of erythrocytes following inoculation of scrapings from cultures of pre-erythrocytic stages. These erythrocytic forms were accompanied by development of gametocytes which in turn were infective for *A. aegypti* mosquitoes. One possible hypothetical explanation for the difference between our results and those of Lewert might be found in the fact that his inoculum contained phanerozoites, while ours contained cryptozoites and metacryptozoites. Cryptozoites in general may have a greater proclivity for invading erythrocytes, while phanerozoites may prefer to remain within the tissue elements.

SUMMARY

The present paper presents the results of investigations dealing with the cultivation of exoerythrocytic forms of *Plasmodium gallinaceum* in tissue cultures prepared from infected chick embryos.

Exoerythrocytic forms were cultivated in cultures derived from spleen, brain, liver, lung, bone marrow, skeletal muscle and heart. Best results were obtained with spleen, brain and liver.

Such forms were found in all stages of development within macrophages, endothelial cells and fibroblasts, in that order of frequency. Partial development of such forms was also observed in epithelium derived from lung implants.

In our experience, the most suitable method for the study of the metabolism of exoerythrocytic forms in tissue cultures proved to be the use of macrophage cultures (derived from splenic implants) cultivated on coverslips without the use of plasma clot. The effects of various environmental factors on the cells and parasites were studied with such a system. Some of the more important factors studied were: the range of hydrogen ion concentration and osmotic pressure tolerated by the cells and parasites; the effects of a heterologous medium, of chick embryo juice, and of concentration of serum; effects of various concentrations of glucose, calcium, inorganic phosphorus, bicarbonate; and the antibiotics penicillin and steptomycin.

Attempts to cultivate the tissues and parasites in synthetic mediums were discouraging.

The sporozoites of *P. gallinaceum* developed into cryptozoites in vitro following the inoculation of salivary glands of infected *Aëdes aegypti* mosquitoes into cultures of normal chicken macrophages. Scrapings from such cultures of pre-erythrocytic stages were infective for chicks and produced a normal life cycle pattern. The majority of such chicks survived the high parasitemia which followed, in contrast to the usual high mortality rate of sporozoite-induced infections in chicks. This difference may have been due to some alteration of the pre-erythrocytic stages in vitro.

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STUDIES ON ENHANCEMENT OF BACTERIAL INFECTION BY HOG GASTRIC MUCIN

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Previous studies[1] have shown that the enhancement of bacterial infection by hog gastric mucin can be antagonized by α-tocopherol or mucin itself, provided the latter are injected at appropriate intervals before the injection of the mucin-bacterium mixture. One explanation for these observations can be based on an increase in capillary permeability,[2] thereby "preparing" the peritoneal cavity to resist the subsequent injection of the mucin-bacterium mixture by local mobilization of plasma and cellular elements of the blood.

It is of interest to report that when mucin or α-tocopherol was injected intraperitoneally into mice at the same time that a vital stain was injected intravenously, more dye was recovered from peritoneal exudate than was the case when neither agent was used intraperitoneally. On the other hand, pretreatment of animals intraperitoneally with mucin or α-tocopherol caused greater absolute amounts of dye to appear in the exudates, irrespective of whether the dye was subsequently given with or without mucin. The action in terms of mean time to death of small amounts of tetanus toxin was enhanced by mucin, whereas the action of larger amounts of toxin was actually inhibited by the use of mucin.

Received for publication December 13, 1951.

Aided by a grant from the University Board of Research.

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1. Elberg, S. and Gale, D. 1949, J. Infect. Dis. **85**: 304–306.

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METHODS

The mucin suspensions were prepared by adding 5.5 g Wilson 1701W hog gastric granular mucin (lot #72817) to 94.5 ml distilled water. This was allowed to stand for approximately 15 to 20 minutes before mixing in a Waring blendor. This procedure allowed the mucin to adsorb water and to form a gummy, viscous mass. Homogenization was accomplished within five to ten minutes by means of the blendor. The stable mucin suspension was sterilized at 15 pounds pressure (121 C) for 15 minutes. The pH was adjusted to 7.0 with sterile $N/10$ sodium hydroxide just prior to use. Mucin suspensions which have not been neutralized remain stable for one week. However, neutralized mucin suspensions aggregate within two days after preparation, and were not used for animal inoculation.

Groups of mice were inoculated intraperitoneally with 0.5 ml of 5% hog gastric mucin or with α-tocopherol (5 mg). Two to three minutes later, the dilated tail veins were injected with 0.5 ml 2% trypan blue solution which had been sterilized by Seitz filtration. Twenty minutes later the mice were sacrificed, either by vertebral compression or by electrocution, and 0.3 ml of the peritoneal exudates removed with capillary pipettes. These aliquots were diluted to a final volume of 6.0 ml with saline and centrifuged at high speed (17,000 G) for ten minutes. The colored supernates were decanted and read for light transmittance in the Coleman spectrophotometer at a wave length of 580 mμ, a value based on the maximum absorption on a spectral transmitance calibration curve (table 1). As controls, mice were injected with gel-diluent (0.1% gelatine added to physiological saline) intraperitoneally and 0.5 ml of the 2% trypan blue solution intravenously, and the peritoneal fluid studied as described.

The 20-minute time interval was chosen to insure sufficient fluid in the peritoneal cavity of the control animals, so that 0.3 ml of exudate could be removed. When mucin was used, the exudates from control animals were suspended in a 1:20 dilution of mucin so that possible adsorption of dye to mucin after centrifugation would be equivalent in both test and control series. If hemorrhage occurred in the peritoneal

TABLE 1.—*Absorption spectrum of a Seitz-filtered trypan blue solution.*

Wave length in mμ	Percent transmittance
400	77.0
425	76.5
450	75.5
475	71.0
500	62.2
525	49.8
550	36.0
570	30.1
575	29.9
580	29.1
585	29.2
590	29.9
595	30.1
600	31.2
625	40.2
650	54.0

cavity, due to either the method of sacrifice or to undue capillary fragility, or if blood cells appeared in the exudate, the material was discarded.

The procedure using α-tocopherol acetate was necessarily more complicated. A group of mice was injected intraperitoneally with 5.0 mg α-tocopherol acetate in 0.2 ml peanut oil and 0.5 ml 2% trypan blue, intravenously, within one to two minutes. Five minutes later, 1.0 ml saline was inoculated intraperitoneally. Fifteen minutes later, the mice were sacrificed by electrocution and 0.3 ml peritoneal exudate was removed. This was diluted to 6.0 ml with saline, centrifuged at high speed, and the supernates decanted. Because the supernates contained oil in varying amounts, 0.5 ml chloroform was added to each tube and the tubes vigorously shaken. The chloroform extracted the oil but left the dye in the aqueous layer. The colored, aqueous, oil-free upper layer was siphoned off into a cuvette and read at 580 mμ. By use of the proper calibration curve the readings were converted to milligrams of trypan blue. Two control series were run: (a) peanut oil without tocopherol and (b) gel-diluent.

TABLE 2.—*Enhanced appearance of trypan blue in peritoneal cavity after intravenous injection of dye.*

	Mg $\times 10^{-4}$ of trypan blue of animals injected with			
	Mucin	Gel-diluent	α-tocopherol acetate	Peanut oil
	73	32	77	24
	60	28	65	53
			79	33
	71	36		
	57	35	75	29
			70	42
	63	26	63	33
	50	32		
	42	24		
	61	29		
	42	33		
	76	24		
	65	24		
	82	33		
	70			
Mean	58	27.4	61.3	30.6
Standard deviation	12.5	4.8	11.2	9.8

The gel-diluent control series which was run at the same time as the α-tocopherol acetate and peanut oil series gave a mean of 23.4 with a standard deviation of 6.9.

RESULTS

The results are summarized in table 2. It will be observed that much more dye appeared in the exudates of mice injected intraperitoneally with either mucin or α-tocopherol and intravenously with dye than in those of the control animals receiving neither mucin nor tocopherol.

The effect of pretreatment by α-tocopherol or mucin intraperitoneally on the subsequent injection of mucin-dye mixtures

Groups of mice were injected intraperitoneally with either 5.0 mg tocopherol acetate in 0.2 ml peanut oil or with 1.0 ml 5% mucin. Forty-five to fifty hours later, half of each group of mice was inoculated with 1.0 ml mucin intraperitoneally, followed in one to two minutes by 0.5 ml 2% trypan blue intravenously. The other half received 1.0 ml gel-diluent intraperitoneally and the dye intravenously. Peritoneal exudates were analyzed as described previously.

The results are presented in table 3. In each series the exudates of the mucin-injected mice contained approximately as much dye as the exudates of the controls. Significantly more dye occurred in the exudates of mice pretreated with either tocopherol or mucin than in those of non-pretreated animals (cf. Table 2).

The data were analyzed by either the significance of the difference between two means, or by Chi square for the significance between two percentages.

TABLE 3.—*The effect of pretreatment with either α-tocopherol acetate or mucin on the appearance of trypan blue in the peritoneal cavity.*

Mucin-pretreated mice		Tocopherol-pretreated mice	
Mg. ×10⁻⁴ trypan blue in exudates		Mg. ×10⁻⁴ trypan blue in exudates	
Mucin injected	Gel-diluent injected	Mucin injected	Gil-diluent injected
93	122	138	108
98	107	134	108
78	91	140	114
115	69	112	120
83	69	118	119
127	118		
109	141		
108	121		
107	133		
111	111		
93	129		
	104		
Mean* 93.5	101.1	107	94.8
Standard deviation 16.2	23.8	24.2	19.5

* Based on analysis for small samples.

The Student *t* value as a measure of the significance between two means was used because the samples used were relatively small.[3]

Several observations which are both interesting and provocative may be made from the statistical analysis of the data of tables 2 and 3 (table 4). A significantly greater amount of dye was found in peritoneal exudates of mucin-injected animals than in the exudates of gel-diluent controls after intravenous inoculation of dye. Alpha-tocopherol acetate injected intraperitoneally showed a very similar effect which could not be attributed to the peanut oil carrier. However, very little difference could be seen between the concentration of dye in the peritoneal exudates of mice which had been inoculated with either mucin or tocopherol.

When mice were pretreated with tocopherol, the same concentration of dye was found in the peritoneal exudates of both mucin and gel-diluent control series; pretreatment with mucin produced the same result. The concentration of dye in the exudates of mice which had been pretreated with either mucin or tocopherol was significantly greater than the amount of dye in the exudates of non-pretreated mice.

Absorption of dye from the peritoneal cavity in the presence of mucin

Since it had been demonstrated that dye injected intravenously appeared more readily in the peritoneal exudate of mucin-injected mice than in that of controls, it was of interest to determine the effect of mucin on the absorption of dye from the peritoneal cavity into the blood stream. In several experiments in

TABLE 4.—*Significance of results on enhancement of appearance of dye in peritoneal cavity.*

Treatment under comparison	Mean	Degrees of freedom	*P*
1. No pretreatment			
Mucin	58.0	25	<0.001
Gelatin-diluent	27.4		
Tocopherol	61.3	12	<0.001
Gelatin-diluent	23.4		
Tocopherol	61.3	12	<0.001
Peanut oil	30.6		
Peanut oil	30.6	12	0.18
Gelatin-diluent	23.4		
Mucin	58.0	19	0.60
Tocopherol	61.3		
2. Tocopherol pretreated	107.0	10	0.36
Gelatin-diluent	94.8		
3. Mucin pretreated			
Mucin	93.5	23	0.31
Gelatin-diluent	101.1		
4. Tocopheral pretreated			
Mucin	107.0	18	<0.001
No pretreatment—mucin	58.0		
5. Tocopherol pretreated			
Gelatin-diluent	94.8	17	<0.001
No pretreatment—gelatin-diluent	27.4		
6. Mucin pretreated—mucin	93.5	24	<0.001
Not treated—mucin	58.0		
7. Mucin pretreated—gelatin-diluent	101.1	24	<0.001
Not pretreated—gelatin-diluent	27.4		
8. Tocopherol pretreated—mucin	107.0	16	0.19
Mucin pretreated—mucin	93.5		
9. Tocopherol pretreated—gelatin-diluent	94.8	17	0.60
Mucin pretreated—gelatin-diluent	101.1		

3. Fisher, R. A. 1948, Statistical Methods for Research Workers, ed. 10, 1948, Edinburgh, Scotland, Oliver and Boyd, pp. 86, 123–124.

which dye had been injected intraperitoneally together with mucin into mice, and blood samples taken at various intervals after inoculation, it was found that comparatively little dye appeared in the blood stream until three hours following the inoculation. In contrast to this, when the dye was injected intraperitoneally with gel-diluent, considerable dye appeared in the blood stream one hour after inoculation.

This work was repeated and extended, with two additional controls included. A series was run with 4% kaolin to control for adsorption of dye by mucin, because this concentration of kaolin had the same adsorptive capacity as 5% mucin when tested in vitro with various dilutions of dye. Another series was run with 12% gelatine, since gelatine in this concentration had approximately the same relative viscosity as 5% mucin when measured in an Ostwald viscosimeter.

Series of mice were injected intraperitoneally with 1.0 ml 5% mucin, 1.0 ml gel-diluent, 1.0 ml 4% kaolin, or 1.0 ml 12% gelatine, respectively, together with 0.5 ml 2% trypan blue. At 20-minute intervals for the first three hours, then at 30-minute intervals to five hours, and at seven hours after inoculation, the mice were sacrificed by cutting their jugular veins. The blood samples (0.2 ml) were quickly diluted with saline to 6.0 ml volume, centrifuged at high speed for ten minutes, and the colored plasmas decanted.

Mucin and 12% gelatine inhibited the passage of dye from the peritoneal cavity to the blood stream for about three hours after inoculation. The effect was more pronounced with gelatine than with mucin. Kaolin seemed very active physiologically, since considerable dye was apparent in the blood stream even 20 minutes after inoculation.

The results described above may be explained in terms of deposition of some "barrier" substance following alteration of the mechanism governing passage of fluid elements into and out of the vascular system. It is also conceivable that the solubility of the dye in the dilute gelatine solution was greater than in the mucin or more concentrated gelatine solutions, the effect being that increased solubility aided absorption. In the case of a less soluble dye preparation clearance from the peritoneal cavity would be slower.

The question as to whether the increase in dye concentration in the peritoneal exudate[4] is merely due to adsorption of dye onto the mucin particles residing therein was considered. Equilibrium resulting from diffusion of small dye molecules into as well as out of the capillaries in the peritoneum may therefore be shifted so that more dye would diffuse out of the capillaries than back into them.

Evidence against adsorption as the basis for increased dye concentration in the exudate of mucin-injected mice is, however, available in the work on pretreatment described above. A first injection of mucin about 45 to 50 hours previously had so altered the host physiology that in mice receiving gelatin-diluent plus dye subsequently the concentration of dye in the exudate was increased over that of non-pretreated controls which had been injected with gel-diluent plus dye. All the mucin had been adsorbed within 24 hours after inoculation, as indicated by examination of stained smears of peritoneal exudate at various time intervals after inoculation of mucin and organisms.*

Groups of mice were inoculated intraperitoneally with gel-diluent, 4400

4. Menkin, V. and Menkin, M. F. 1930, J. Exper. Med. 51: 285.

* Unpublished work.

Salmonella typhi, or mucin plus 4400 *S. typhi*, respectively. In addition, groups of mice were inoculated intraperitoneally with 5.0 mg α-tocopherol acetate in 0.2 ml peanut oil, or 1.0 ml mucin, and 36 hours later with mucin plus 3300 organisms. At the various time intervals indicated in table 5, one mouse in each series was sacrificed by vertebral compression, smears of the peritoneal exudate prepared, stained by carboltoluidine blue, and 100 cells differentiated as to type and phagocytic activity. The percentages of the polymorphonuclear cells at each time interval and for each series, and the results of comparisons between the controls and the test series by means of Chi square, are presented in table 5.

TABLE 5.—*The cellular response to mucin in the peritoneal cavity of mice pretreated with α-tocopherol acetate or mucin.*

Hours after noculation	No pretreatment					Tocopherol pretreated			Mucin pretreated		
	Gel-diluent	4400 *S. typhi*		Mucin plus 4400 organisms		Mucin plus 3300 organisms			Mucin plus 3300 organisms		
	Percent poly's	Percent poly's	P_1*	Percent poly's	P_1*	Percent poly's	P_1*	P_2†	Percent poly's	P_1*	P_2†
2	19	20	0.85	5	<0.001	58	<0.001	<0.001	78	<0.001	<0.001
3	40	37	0.71	10	<0.001	68	<0.001	<0.001	80	<0.001	<0.001
4	33	33	1.00	24	0.24	88	<0.001	<0.001	82	<0.001	<0.001
8	18	25	0.28	83	<0.001	93	<0.001	0.51	85	<0.001	0.87
24	12	11	0.83	95	<0.001	89	<0.001	0.67	95	<0.001	1.00

P is the probability as determined by Chi square with one degree of freedom[3] that the difference between the two percentages being compared may be due to chance. If P is less than 0.05, then the difference between the percentages is significant.
* P_1 is the result of the comparison to the gel-diluent control.
† P_2 is the result of the comparison to the mucin plus 4400 organisms series.

The cellular response in animals receiving either the diluent or organisms alone was so similar that the comparisons were made to the diluent controls. Significantly few leukocytes were seen for the first three hours following injection in the mucin-inoculated mice, but thereafter, the polymorphonuclear response was massive—indicative of a marked inflammatory reaction or an increase in capillary permeability. In the pretreated animals, the enhanced leukocytic response was apparent, even for the first three hours following the subsequent inoculation of the mucin. This can be interpreted as evidence that the pretreatment because of the inflammatory reaction or because of altered capillary permeability antagonized for three hours the inhibition of leukocytic migration which was exerted by the mucin.

Effect of mucin on tetanal intoxication

Groups of 15 mice were injected with varying amounts of tetanal toxin* plus either 0.5 ml of 5% mucin or 0.1% gelatine in 0.85% saline. The time of death of the animals was determined as closely as possible and the data and their analyses are presented in tables 6 and 7. For each dilution of toxin mucin effected a larger number of fatalities than gelatine diluent for the time interval studied. On the other hand, measurements of mean time of death showed that the

* The tetanus toxins and antitoxins were generously supplied by Cutter Laboratories. The antitoxin (lot #C5191) was a concentrated, enzyme-digested globulin which contained 7600 units per ml. The toxin (lot #3734) contained 100,000 MLD for guinea pigs, subcutaneously, and 100,000 MLD for mice intraperitoneally. The L+ dose was determined by finding the amount of toxin in a 0.5 ml volume which when injected intraperitoneally would kill 4 out of 5 mice which had received 1.0 unit antitoxin, intravenously, within 18 hours. The L+ dose for these lots of toxin and antitoxin was found to be a 1:50 dilution.

TABLE 6.—*The effect of mucin on the death of mice injected intraperitoneally with dilute tetanus toxin.*

L+dose of toxin	Hours after inoculation	Mucin series		Control series		Chi square*	P
		Number dead	Percent dead	Number dead	Percent dead		
0.0333	16	7/15	46.5	2/15	13.3	18.1	<0.001
0.0083	24	15/15	100.0	2/15	13.3	67.0	<0.001
0.0033	37	13/15	86.6	8/15	53.3	7.7	<0.001
0.0017	46	13/15	86.6	6/15	40.0	16.8	<0.001
0.0008	67	11/15	73.4	7/15	46.5	6.1	<0.001

* Chi square determined according to equation on the lower part of p. 86,[3] with one degree of freedom.

most significant mucin effect occurred when 0.008 L+ units of toxin were used and limiting effects when 0.0017 L+ and 0.0008 L+ units were used.

Two explanations which suggest themselves for the faster death rate in the mucin-treated animals given dilute toxin are that the mucin aided the absorption of the soluble toxin from the peritoneal cavity and/or that the mucin "potentiated" the toxin (see below). Since there are so few motor nerve endings in the peritoneum, the other pathways by which the toxin would be absorbed from the peritoneal cavity would be the lymphatics and blood stream. If an increase in the permeability of the capillaries occurred as a result of mucin injection, the toxin would be able to leave the peritoneal cavity and reach its site of action earlier.

Since the intraperitoneal inoculation of mucin with small amounts of toxin seemed to aid the absorption of the toxin from the peritoneal cavity, the question arose as to what effect the mucin might have on large amounts of toxin. This work was performed with groups of ten mice each and various amounts of toxin given intraperitoneally with mucin or diluent. Figure 1 and table 8 present different aspects of the results.

Figure 1 is a graphical representation of the cumulative number of dead for each dilution of toxin at various time intervals after inoculation. The differences in the time of death of the mucin-treated animals and the controls are very clearly shown. For the greater dilutions of toxin, the differences become increasingly smaller, until at 0.8 to 0.5 L+ units the curves of each series overlap to a considerable extent. The statistical analysis of these data is presented in table 8. The mean time of death in the mucin-treated mice was significantly greater than that in the controls, for concentrations of 5.00 to 1.67 L+ units. The differences between the mean times of death in both series for the smaller amounts of toxin were not significant. One can infer from these data that a concentration of toxin could

TABLE 7.—*Significance of the difference in mean time of death of mice intraperitoneally injected with dilute tetanus toxin plus mucin or diluent.*

L+dose* of toxin	Mucin		Gel-diluent		Estimated† standard error	t	Degrees of freedom†	P
	Mean time† of death (hours)	Standard deviation (hours)	Mean time† of death (hours)	Standard deviation (hours)				
0.0333	17½	2.4	18½	3.3	2.9	1.186	30	0.28
0.0083	22	2.0	27½	2.9	3.0	4.900	29	<0.001
0.0033	33‡	3.2	34‡	4.0	3.6	1.361	29	0.20
0.0017	40‡‡	5.6	46	5.6	5.6	2.750	29	0.01
0.0008	61‡‡	7.4	68‡	7.7	7.5	2.053	29	0.05

* L+ dose determined as described in the text.
† Computed according to the equations on p. 123.[3]
‡ Fourteen mice; all other groups, 15 mice.

TABLE 8.—*The effect of mucin on the toxicity of relatively large amounts of intraperitoneal tetanus toxin.*

L+ dose of toxin	Mucin		Gel-diluent		P
	Mean time of death (hours)	Standard deviation (hours)	Mean time of death (hours)	Standard deviation (hours)	
50*	7	1.9	2¼	1.2	0.002
10*	10	3.5	4	1.0	0.008
5.00	11	2.6	6	1.7	<0.001
3.3*	12½	4.6	6¼	1.5	0.02
2.50	13½	3.2	7½	1.5	<0.001
2.0*	12½	5.5	7½	2.6	0.09
1.67	15½	4.4	8	1.2	<0.001
1.20	10	2.3	8¼	2.2	0.20
1.00	10¼	1.9	8¼	1.3	0.05
0.83	10¾	1.8	10¼	1.5	0.50
0.50	11¾	1.6	10¾	1.5	0.28

* Five mice per group; 10 mice per group for all others.

be determined, probably within the range of 0.83 to 0.33 L+ doses (mucin vs. control), at which the mean time of death in both series would be the same.

At least two explanations for the delay in the action of larger amounts of toxin in mucin-treated animals can be offered. One is that the toxin might have been partially detoxified by the mucin, and thus would not kill these animals as readily as it would the controls. The data in table 7 for dilutions of toxin greater than 5.0 to 1.2 L+ doses speak against such an explanation. Moreover, evidence was obtained that detoxification of toxin in the presence of mucin does not occur (see below).

Another explanation would be that the mucin, either mechanically as by the viscosity factor,[5] or by the formation of a "barrier" of some type, holds the toxin within the peritoneal cavity for a period of time. Thereafter, the toxin is released to reach its site of action and produce the toxemia and death of the animal. An estimation of the time that such a "barrier" might exist can be calculated from the data as follows:

If the standard deviation is subtracted from each of its accompanying means, the value obtained indicates the minimal time at which that concentration of toxin would produce death of the animal. If this minimal time of death in the controls is subtracted from the minimal time of death of the mucin-treated mice, the figures obtained reflect the minimal time difference between the two series. When these time differences for the amounts of toxin, from 5.0 to 1.67 L+ doses are averaged (since the effect was observed only at these concentrations), the mean obtained is $3\frac{1}{2}$ hours, with a standard deviation of $\frac{3}{4}$ hour. Hence the barrier should be in existence around three to four hours. This has been histologically verified and will be the subject of another report.

The influence of mucin on the absorption of toxin from the peritoneal cavity can be observed in figure 2, in which the mean times of death for the various concentrations of toxin in the mucin and control series are plotted against time. There are three distinct areas on these curves which are of particular interest: (1) Area A:—The mucin-injected animals died significantly later than the controls. (2) Area B:—The mice which received mucin died at relatively the same time as the controls. (3) Area C:—The mucin-treated mice died more rapidly than the controls.

It is postulated here that increase in capillary permeability is demonstrated

5. Smith, H. 1950, Biochem. J. **46**: 352–356; 356–363; 1951, **48**: 441–447.

by area C (fig. 2) and the formation of a "barrier" by area A. The time at which the "barrier" is being dissolved or destroyed (in some fashion as yet unknown), and at which the increase in capillary permeability is becoming dominant, would be area B.

The formation of a "barrier" apparently occurred equally as well in mice inoculated with dilute toxin as with con-

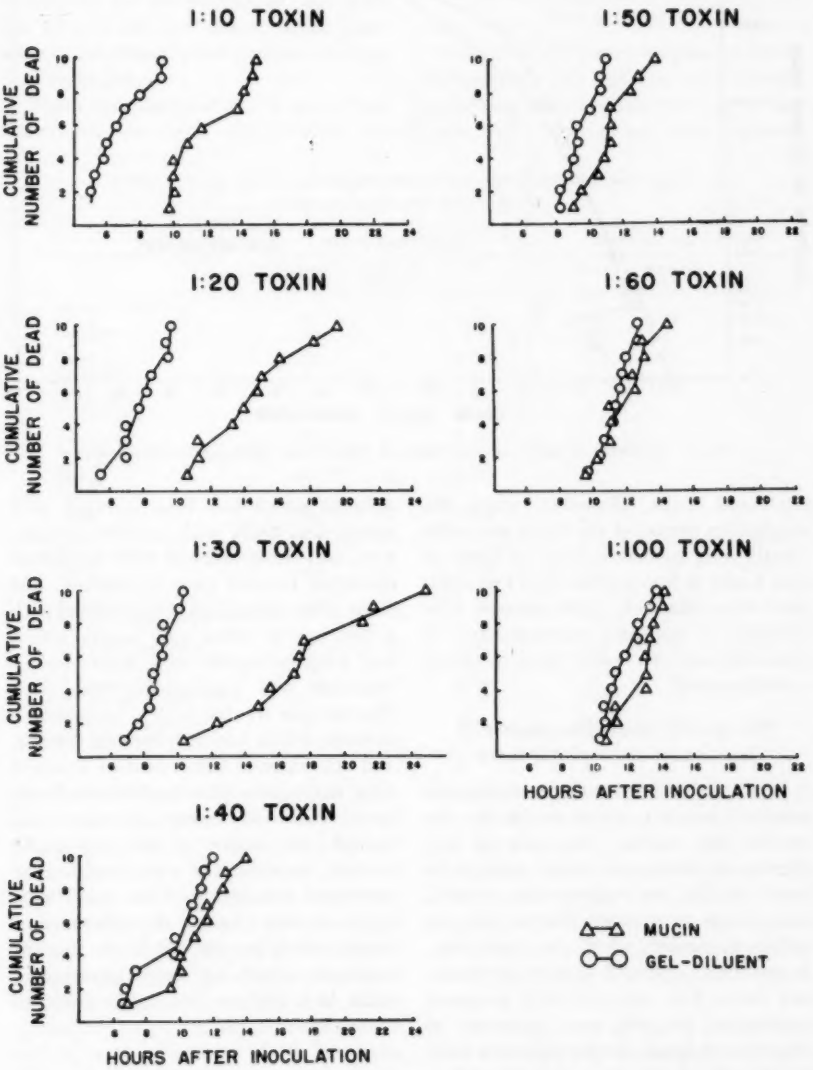

FIG. 1.—Effect of mucin on lethality of large amounts of tetanal toxin.

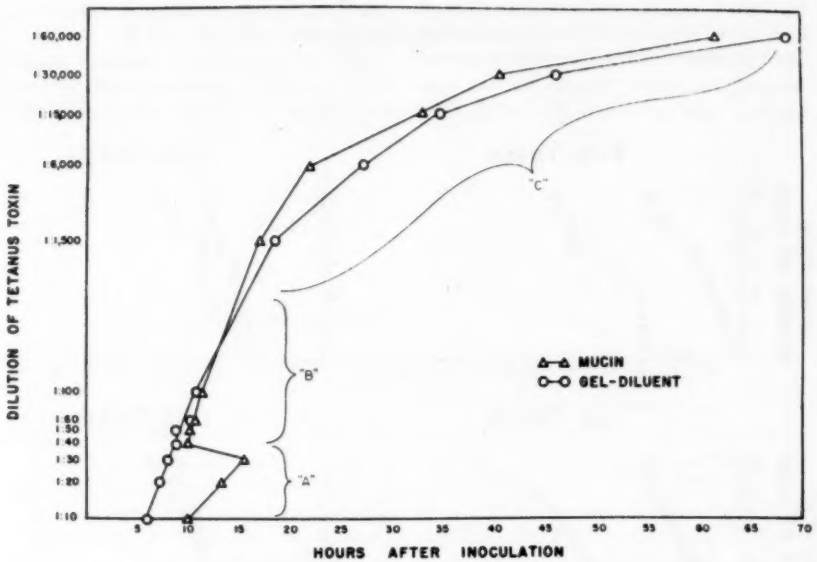

FIG. 2.—Influence of mucin on absorption of tetanal toxin from peritoneal cavity.

centrated toxin. However, when the incubation period of the toxin was sufficiently long so that a delay of three to four hours in leaving the site of inoculation was relatively unimportant, the increase in capillary permeability as demonstrated by faster time of death was observed.

The effect of in vitro incubation of mucin with concentrated toxin

It became of interest to determine whether tetanus toxin could be detoxified by mucin. Mixtures of 1:5 dilution of toxin and mucin were incubated at 37 C for various time periods, and groups of nine mice each injected intraperitoneally with the mixtures. In addition, a control mixture of mucin and toxin was injected with no prior incubation as well as a mixture of diluent and toxin. As the mixtures were incubated for a longer period, the difference in mean time of death between the animals which had been injected with mucin and toxin with no prior incubation, and those injected with incubated mixtures, became more significant. The mean time of death of mice injected with a mixture of toxin and mucin which had been incubated nine hours before injection was significantly less than that of mice treated with a toxin-mucin mixture which had not been incubated, and approximated that of mice injected with toxin plus diluent which had also been incubated before injection. Although potentiation[6] of the toxin might account for this, it is also possible that prolonged incubation of the toxin with mucin caused a loss of the substance in mucin which is responsible for the antagonistic action on larger amounts of toxin. It is evident that mucin does not cause detoxification.

6. Zuger, B., Hollander, A. and Friedemann, U. 1939, J. Infect. Dis. **65**: 86–91.

The effect of α-tocopherol acetate on tetanus toxin injected intraperitoneally

Groups of mice were inoculated intraperitoneally with 5.0 mg α-tocopherol acetate in 0.2 ml peanut oil and with 0.5 ml 1:5 (10 L+ doses) toxin. Controls of gel-diluent, and peanut oil, were also included.

Alpha-tocopherol delayed the lethal action of the toxin significantly (at $P = 0.05$ level) as compared to the gelatin-diluent controls, but was not as effective as mucin. Peanut oil also delayed the action of the toxin, but its effect was not significantly different from the diluent controls. Since the mean time of death in the tocopherol series was not significantly different from that of the peanut oil controls, the delay in the action of the toxin could be referable to the viscosity of the preparation rather than to any specific activity of the α-tocopherol as conclusively shown for fractions of mucin.[5]

The effect of preparation of animals with α-tocopherol acetate or mucin on their response to mucin-toxin mixtures

Groups of mice were inoculated intraperitoneally with 5.0 mg α-tocopherol acetate in 0.2 ml peanut oil. Forty-two hours later one group was injected intraperitoneally with 0.5 ml 1:5 (10 L+ doses) toxin and 0.5 ml mucin, and another group with toxin and 0.5 ml diluent. The third and fourth groups were inoculated intraperitoneally with 0.5 ml 1:60,000 (0.0008 L+ dose) toxin and 0.5 ml mucin or diluent, respectively, 51 hours after the tocopherol inoculation.

The mean time of death of prepared mice subsequently inoculated with mucin and 10 L+ units toxin was signifi-

TABLE 9.—*The effect of pretreatment with α-tocopherol acetate on the response of mice to mucin plus tetanus toxin.*

Treatment under comparison	10 L+ doses of toxin			0.0008 L+ doses of toxin		
	Mean (hours)	Degree of freedom	*P*	Mean	Degree of freedom	*P*
1. No pretreatment						
Mucin	10	10	0.008	61½	29	0.05
Gel-diluent	4			68½		
Tocopherol	6½	14	0.027			
Gel-diluent	4					
Tocopherol	6½	14	0.50			
Peanut oil	5½					
Peanut oil	5½	10	0.15			
Gel-diluent	4					
Mucin	10	14	0.022			
Tocopherol	6½					
Mucin	10	10	0.022			
Peanut oil	5½					
2. Tocopherol pretreated						
Mucin	9½	20	0.008	57	13	0.92
Gel-diluent	6			57½		
Pretreated—mucin	9½	15	0.92	57	19	0.27
Not pretreated—mucin	10			61½		
Pretreated—gel-diluent	6	15	0.06	57½	23	0.001
Not pretreated—gel-diluent	4			68½		
Pretreated—mucin	9½	15	<0.001	57	20	0.014
Not pretreated—gel-diluent	4			68½		
Pretreated—gel-diluent	6	15	0.01	57½	22	0.23
Not pretreated—mucin	10			61½		

cantly later than that of prepared animals injected with toxin alone, but differed very little from the time of death of mice which had received mucin and toxin with no preparation (table 9). The prepared mice subsequently injected with toxin alone showed an appreciably later time of death than the nonprepared controls (significant at the 5% level). With 0.0008 L+ unit toxin, the results were considerably different. Prepared mice inoculated with either mucin-toxin or toxin alone gave the same mean time of death. This was significantly less than that of nonprepared controls (which had received only toxin), but not much less than that of nonprepared mice which had been injected with mucin and toxin. Preparation of animals with mucin instead of tocopherol and subsequent injection of mucin-toxin or diluent-toxin mixtures gave similar results.

Pretreatment with either α-tocopherol acetate or mucin showed a very similar effect on mice injected intraperitoneally with dilute toxin, but unlike the results obtained with the dye studies, the effect of pretreatment was not significantly different from that produced by mucin with no pretreatment. Pretreatment had no demonstrable augmenting effect on the subsequent inoculation of concentrated toxin with mucin and delayed the action of concentrated toxin when used alone.

DISCUSSION

The precise measurement of variables affecting dispersal of toxins, dyes and bacteria is difficult at best, and the subject has been well explored by others.[8–12] Many differences in tissue-blood exchange which have been interpreted as evidence for altered capillary permeability may be due to fluctuations in hydrostatic pressure, factors regulating peripheral blood flow, or avidity of extracapillary tissues for either solute or solvents.[10] Intercellular cement substance may act as a selective filter, dependent on variations in its porosity which could be affected by the other components of the structure of the capillary wall. Changes in capillary permeability would be the result of responses of any or all of the components of the wall structure.

The most common technique for demonstration of altered capillary permeability is the examination of the effect of agents on the amount of dye exuded from the blood stream into a lesion. Since the inflammatory reaction is complex, the reliability of data on increased exudation of dye as a reflection of altered capillary permeability may be questionable. Moreover, stainability of the tissues by the dye and absorption of dye to blood proteins must also be considered. Miles and Niven[13] observed that "transient mechanical interference with the blood supply of the skin can produce changes similar to those following changes in capillary permeability."

The dye method used in this work was not subject to these limitations. Stainability of the cells and mucin in the peritoneal exudate was of little importance, since the diluted dye measured spectrophotometrically was the free dye in the supernate after centrifugation of

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10. Chambers, R. and Zweifach, B. W. 1947, Physiol. Rev. **27**: 436–463.
11. Miller, R. G. 1938, J. Exper. Med. **67**: 619–641.
12. Barnes, J. M. and Trueta, J. 1941, Lancet, **1**: 623–626.
13. Miles, A. A. and Niven, J. S. F. 1950, Brit. J. Exper. Path. **31**: 73–95.

the exudate. Little inflammatory reaction was manifested in the short interval between the intraperitoneal inoculation and the intravenous dye injection, or in the 20-minute interval thereafter before the exudate was removed. The problem of adsorption of dye by the mucin was not a factor of importance. The data on the enhanced exudation of dye into the peritoneal cavity reported above can be interpreted as evidence for increased capillary permeability induced by mucin or tocopherol. It is of interest that Menkin[4] based his early finding of increased capillary permeability in inflammation upon a similar technique which differed only in that loss of dye from the capillaries into the peritoneal cavity rather than increased exudation of dye was measured.

The existence of a mechanism opposing the dispersal from the peritoneal cavity for a few hours after injection of mucin was based on the following observations, the first of which is to be reported in detail elsewhere: (1) the demonstration of fibrin granules either on the outside or within the connective tissue layer of the serosal surface of the peritoneal wall for the first three to four hours after the inoculation of mucin and organisms, and the lack of such fibrin in the gelatin-diluent controls; (2) the smaller amount of dye in the blood stream of mice which received dye and mucin intraperitoneally than that in controls, for three hours following inoculation, and possibly referable to dye solubility characteristics; (3) the longer time for death of animals which received high concentrations of tetanus toxin with mucin intraperitoneally than that of controls, the time difference being approximately three to four hours.

Hughes[14] studied filtration under pressure or diffusion through a fibrin barrier in vitro. Although his findings can only simulate in vivo conditions, the results obtained were both interesting and pertinent. He found that trypan blue was held back by the fibrin barrier (a retracted human plasma clot supported on a gradocol membrane) in both filtration and diffusion experiments. Organisms were held back. Bacterial toxins, such as diphtherial and staphylococcal, passed through under high pressure, but did not diffuse through the barrier or pass through at the low pressures expected in vivo. When serum was filtered or allowed to diffuse through the barrier, the globulins were concentrated behind the filter. Leukocytes were able to pass through the fibrin but their passage was inhibited for the first few hours.

It has been shown that tetanus toxin reaches the central nervous system by way of the arterial circulation,[7] and not up the nerve axis cylinders, or by way of the endoneural or perineural lymphatic vessels. A large proportion (80 to 90%) of the toxin was demonstrated in the lymph and blood systems of a sheep which had been inoculated intramuscularly with doses of toxin greater than the MLD. Moreover, a dual action of the toxin was postulated: (1) locally, on the motor nerve endings of the muscle, to produce a local tetanus (muscular rigidity) and (2) centrally, on the anterior horn cells to produce the excitability of the muscles which characterizes general tetanus (anterior horn cells reached via the blood stream and the lymphatic capillaries). The work described above adds confirmation to both postulates. When relatively large amounts of toxin in saline were injected intraperitoneally, generalized tetanus resulted, as manifested by the Cheyne-Stokes breathing, no muscular rigidity, tetanic spasm in the last five minutes

14. Hughes, W. H. 1948, Brit. J. Exper. Path. **29**: 173–180.

before death, and death within 16 hours. With very small amounts of toxin (0.0008 L+ doses or 1 to 2 MLD), the animals showed a muscular rigidity of the peritoneal wall, as well as the generalized tetanus and death within 65 to 70 hours. Since so few motor nerve endings are found in the peritoneal cavity, the toxin in excess of the MLD would be absorbed by the lymphatic vessels and the blood stream and carried to the anterior horn cells to produce the generalized tetanus. The MLD dose would be absorbed to the motor end plates to produce the local tetanus manifested by the muscular rigidity of the peritoneal wall. Since the animals died so rapidly from the large amounts of toxin, because of the generalized tetanus, the local tetanus could not be seen.

That inhibition of leukocyte migration occurs for three hours following inoculation of mucin intraperitoneally has been demonstrated by several workers[15–17] and has been observed as well during the experimental work described herein. Coincident with the inhibition of leukocyte migration, the number of organisms in the peritoneal cavity increased and bacteremia occurred about three hours after the intraperitoneal inoculation, remaining until death of the animals.

Inoculation of α-tocopherol or mucin 36 to 48 hours prior to injection of mucin and organisms (all injections being made intraperitoneally) antagonized the lethal activity of the mucin-organisms mixture.[1] In the experimental work described herein, the data on pretreatment with tocopherol or mucin, as demonstrated with both a dye technique and tetanal toxin studies, are interpreted as indicating increased capillary permeability, thus allowing for adequate dispersal of the toxic agent.

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SUMMARY

1. Measurements of exchange of a vital dye and of tetanus toxin between the blood and the peritoneal cavity have been made in the presence and absence of agents which enhance infection or antagonize such enhancement.

2. The results have been interpreted in terms of the ability of such agents to alter capillary permeability, establish a "barrier" (possibly fibrin) or affect leukocyte migration, depending upon the time of administration of the agent with respect to the time of injecting the indicator substance.

3. Further observations on tetanus pathogenesis were made which are interpreted, with most economy of hypothesis, in terms of toxin transport by the blood and lymph systems.

4. The nature of the effect of mucin on enhancement of tetanal intoxication in mice is governed by the amount of toxin employed: mucin not only fails to enhance the toxicity of large amounts of toxin but delays the time of death. With small amounts of toxin the action of mucin is enhancing in terms of time of death.

CYTOCHEMICAL EVIDENCE FOR THE NATURE OF HERPES SIMPLEX INCLUSION BODIES

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The experiments reported below were designed to test the notion that viral inclusion bodies represent virus colonies and that the inclusion bodies undergo a phase of rapid increase of virus material (and of the enzymatic activities with which it may be associated) followed by a phase of decline. The work was based on the assumption that virus particles of most animal virus diseases contain DNP (desoxyribonucleoproteins). Herpetic inclusion bodies, in different stages of their development, have accordingly been examined by cytochemical methods for their DNP content and for selected enzymatic activities.

MATERIALS AND METHODS

Chorioallantoic membranes of 10- to 13-day-old chickens' embryos were infected with herpes simplex virus. The strain used was Armstrong 1166. The membranes were infected at fixed intervals (twice or once daily) and were harvested together. The time interval between infection of the membranes and harvesting ranged between six hours and six days. The harvested membranes were fixed immediately upon removal and processed simultaneously. Membranes were fixed for four hours in a 5% solution of acetic acid in absolute alcohol at a temperature of −6 to −8 C according to a procedure previously described.[1] After overnight dehydration at room temperature with absolute alcohol, the tissues were passed through two changes of benzene, infiltrated in paraffin at 56 C for about 30 minutes under reduced pressure, and then embedded. Sections, cut at 6 to 8 μ, were stained with haematoxylin-eosin, Giemsa, Feulgen's method with fast green counterstaining,[2] and for four enzymes: alkaline phosphatase[3] and acid phosphastase[4] (Gomori's methods), lipase[3] and Gomori's cholinesterase,[5] without any counterstain. For each staining method sections of all blocks were stained simultaneously in one staining jar.

Received for publication December 14, 1951.

The authors are extremely grateful to Mr. Dov Sulitzeanu of the Laboratory for Virus Research, Dept. of Bacteriology and Hygiene, The Hebrew University, for the preparation of the infected membranes, and to Dr. H. Bernkopf, head of that laboratory, for his help. Thanks are also due to Dr. G. Gomori of the University of Chicago for the supply of myristoyl-choline, and to Mrs. H. Weinman for the microphotographic work.

RESULTS

Hematoxylin-eosin and Giemsa-stained preparations

During the first 18 hours ectodermal proliferation was noted. The nucleoli of the ectodermal cells were enlarged and stained heavily, and in some of the nuclei several nucleolus-like structures could be discerned (fig. 1).

At 24 to 32 hours some shallow ulcerations were noted in the ectoderm and some nuclei were vesicular, had marginated chromatin, and contained slightly or moderately basophilic inclusion bodies. At this stage a proliferative reaction was noted in the entoderm. A nonspecific inflammatory reaction, which subsequently increased in severity, was found in the mesoderm. At 48 to 60 hours the ectodermal prolifera-

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tion and ulceration were marked. Most inclusion bodies were strongly basophilic, with margination or apparent disappearance of the chromatin. Some inclusions were less basophilic, and a few were eosinophilic. At this stage typical inclusion bodies were found also in the mesoderm and entoderm (fig. 2). On the fourth day the ulceration was even more marked in the ectoderm. In the entoderm, besides papillary and flat proliferations, there was also some evidence of ulceration (fig. 3). Most inclusion bodies were eosinophilic and amorphous. Some were very weakly stained and presented an appearance of nuclear vacuoles. A few were basophilic. In some places cells containing inclusions seemed to have fused and formed multi-inclusioned balloons (fig. 4). Occasionally this process was associated with the formation of vacuoles in the ectoderm.

On the fourth, fifth and sixth day, there was evidence of ectodermal regeneration, in the form of multilayered band of cells which grew on the mesodermal surface and was distinctly separated from the overlying, ulcerated, partly necrotic material. At this stage there were very few basophilic inclusions. Most inclusions in the surviving cells were foamy, vacuolated, and lightly eosinophilic. On the sixth day most inclusions were represented by vacuoles with occasional small shreds of chromatin adhering to their periphery (fig. 5). Very few inclusions could be seen in the regenerated ectodermal cells.

Feulgen-stained preparations

In the intranuclear bodies observed during the first 24 hours, no Feulgen-positive material could be discerned, and no distinction could be made between early stages of inclusion bodies and nucleoli.

At 32 hours most inclusion bodies, although filling the nuclei, were Feulgen-negative, or slightly positive. Between this time and 60 hours, the stainability of most inclusion bodies with Feulgen increased progressively, so that at that period most inclusions stained heavily, and were of a fine granular appearance. After this time there was a progressive decrease of the intensity of Feulgen staining of the inclusion bodies, so that on the fifth day there were hardly any which took the stain.

Alkaline and acid phosphatases

The intranuclear bodies of the first 24 hours were indistinguishable from nucleoli, as both reacted heavily. From 24 hours onwards, inclusion bodies reacted with different and progressively increasing intensity (fig. 6). Accurate observation of the intensity of the phosphatase activities of inclusion bodies was difficult, as degenerating cells and necrotic material reacted more strongly than any of the well preserved structures.

Lipase and cholinesterase

No difference between nucleoli and early stages of inclusion bodies could be observed in the sections treated for visualization of these enzymatic activities, as both these structures reacted rather strongly (fig. 7). From 24 hours till 60 hours, both these enzymatic activities of the inclusions increased, reaching a peak, and decreased gradually after the end of this period of time (fig. 8).

It should be noted that all the observations above refer to the bulk of inclusion bodies, while in every single section there was marked variation in the intensity of the various staining reactions.

DISCUSSION

The first visible intranuclear change following infection is the appearance of

an enlarged nucleolus. During the first 24 hours the lumen of the nucleus is filled with a typical inclusion body which appears to have developed from a nucleolus-like inclusion, indistinguishable from the nucleolus by any of the means at our disposal. At this early stage the inclusion body does not contain any visible amount of DNP.

During the period between 24 and 60 hours after inoculation, in our experimental conditions, the inclusion bodies become progressively more basophilic, and the quantity of DNP in them increases similarly. The amount of lipase and cholinesterase per inclusion body increases together with the increase of DNP and basophilia.

After that period there is a progressive diminution of basophilia of the inclusions, which become eosinophilic, then paler, until they eventually lose their stainability. This process is accompanied by a diminution of DNP of these bodies (findings which correspond to previous reports on vaccinia[6] and herpes[7]), as well as of their lipase and cholinesterase contents. The alkaline and acid phosphatases do not show such variations, since their amount increases with cellular damage.

DNP is found in the metazoan cells only in the chromatin substance. It is believed that its presence in forms which bear no resemblance to chromatin should be considered as presumptive evidence for the presence of living matter foreign to the cell, e.g., virus particles.[8] This assumption is supported by the widespread opinion that DNP is an essential constituent of many (all?) animal viruses.

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7. Crouse, H. V., Coriell, L. L., Blank, H. and McNair Scott, T. F. 1950, J. Immunol. **65**: 119–128.
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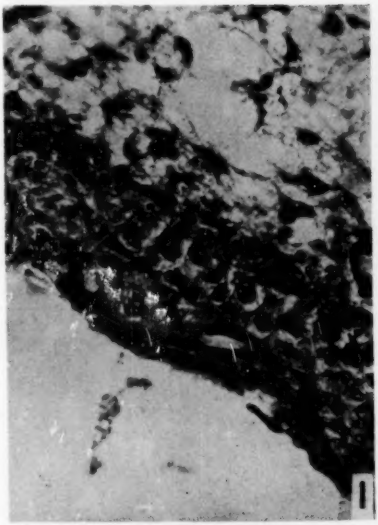

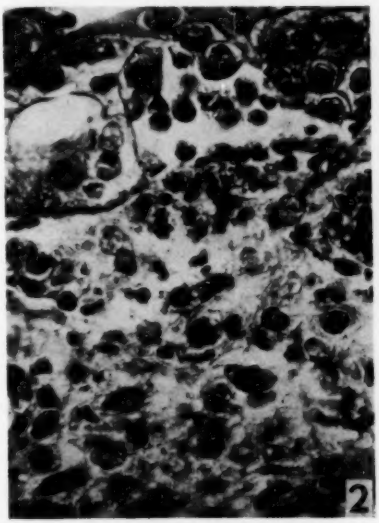

All figures are of chorioallantoic membranes of chick embryos infected with herpes simplex virus.

FIG. 1.—A membrane 24 hours after infection. Early stage of intranuclear inclusion bodies. Feulgen+fast green. ×550.

FIG. 2.—A membrane three days after infection. Ectodermal and mesodermal inclusions. Hematoxylin-eosin. ×550.

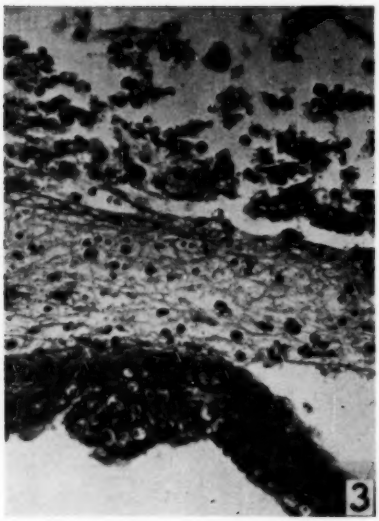

FIG. 3. A membrane four days after infection. Ectodermal ulceration and entodermal cellular proliferation. Hematoxylin-eosin. ×208.

It may be assumed that virus particles possess various enzymatic activities related to their metabolic processes. Whether these enzymes belong to the host, and are utilized by the virus particles to which they adhere, or whether they belong to the virus itself, is beyond the scope of this discussion. Various experimental data obtained by biochemical[9–12] and cytochemical methods[7] show that virus particles contain or adsorb enzymes, or that the multiplication of virus is accompanied by increased enzymatic activities. The finding that two different enzymatic activi-

9. Smadel, J. E. 1948, Viral and Rickettsial Infections of Man, edited by T. M. Rivers, Philadelphia, Lippincott, p. 329.
10. Bauer, D. J. 1947, Brit. J. Exper. Path. **28**: 440–446.
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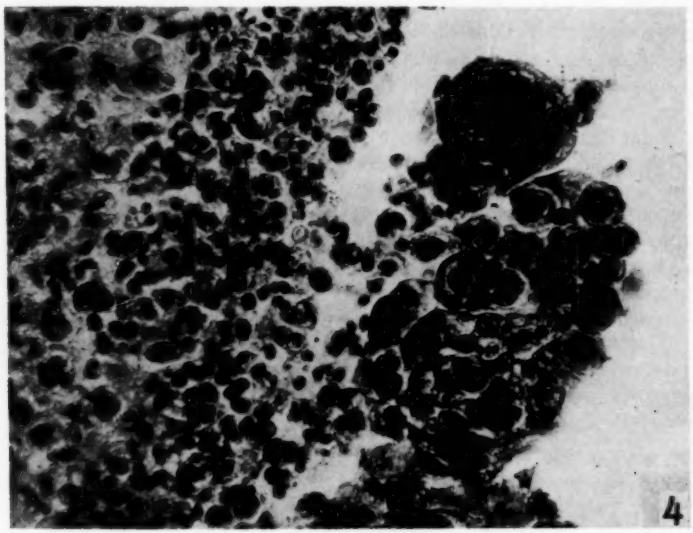

FIG. 4.—A membrane three days after infection. Ectodermal multi-inclusioned balloons. Hematoxylin-eosin. ×550.

ties vary as the quantity of DNP might be considered as additional evidence that the amount of virus in the inclusions varies with their age. However, the value of the observations on the relative amounts of enzymatic activities might be reduced if the recently expressed doubts regarding the specificity of enzymatic cytochemical methods[13,14] and especially those of the phosphatases should prove founded.

The findings are interpreted, therefore, as follows: The early inclusion body is a nucleolus or a nucleolus-like body which contains probably few virus particles, or else does not contain any. The amount of virus per inclusion body increases progressively until it reaches a peak, which in our experiments occurred around 2½ days after infection of the membranes. After the peak, the amount of virus in the inclusion diminishes progressively, until a virus-free, pale eosinophilic inclusion is formed. It is believed that the different descriptions found in the literature for the staining characteristics of inclusions due to one and the same virus might be accounted for by the different ages of inclusions observed by the various investigators.

SUMMARY AND CONCLUSIONS

1. The histological changes in chorioallantoic membranes infected with herpes simplex virus are described. These changes include ectodermal and entodermal proliferations, followed by ulceration and subsequent regeneration of the ectoderm.

2. Inclusion bodies were found in the ectoderm, entoderm and mesoderm.

3. The first appearance of inclusion bodies was in the form of intranuclear bodies indistinguishable from nucleoli,

13. Novikoff, A. B. 1951, Science, **113**: 320–325.
14. Feigin, F., Wolf, A. and Kabat, E. A. 1950, Am. J. Path. **26**: 647–655.

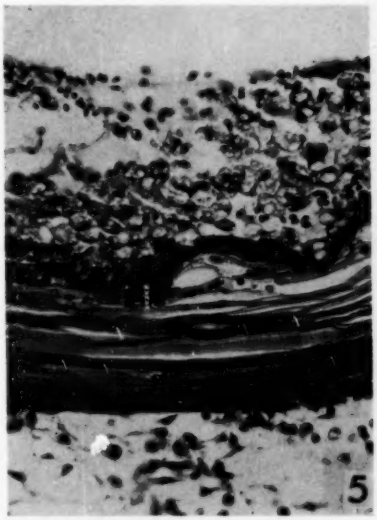

FIG. 5.—A membrane six days after infection. Regenerated ectodermal band underneath ulcerated surface containing vacuolated and foamy nuclei. Hematoxylin-eosin. ×350.

FIG. 6.—A membrane 4½ days after infection. Alkaline phosphatase activity of moderate intensity in inclusions, and of much greater intensity in necrotic matter and debris. Alkaline phosphatase. ×450.

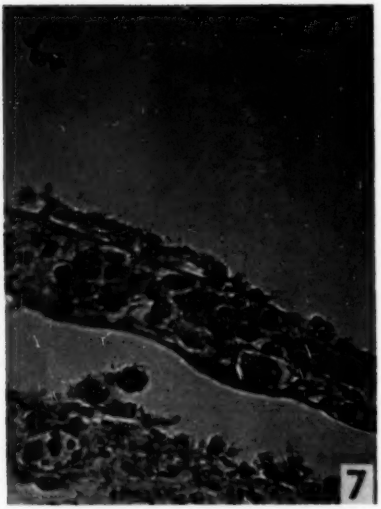

FIG. 7.—A membrane 32 hours after infection. Inclusion bodies. Lipase. ×450.

in which DNP (desoxyribonucleoproteins) could not be visualized. These bodies enlarged and filled the nuclei, and the amounts of DNP, lipase and cholinesterase contained in them, as well as their basophilia, increased in a parallel way till a peak was reached at about 2½ days after the membranes were infected. Afterwards, the inclusions became eosinophilic and then they gradually lost their stainability. At the same time, the amounts of DNP, lipase and cholinesterase decreased until the inclusions were represented by foamy eosinophilic or apparently empty nuclear vacuoles.

4. These findings are considered to be corroborative for a hypothesis that the herpetic inclusion body represents a virus colony inside a cellular matrix, and that the virus particles multiply for a period, after which they slowly disappear from the inclusion.

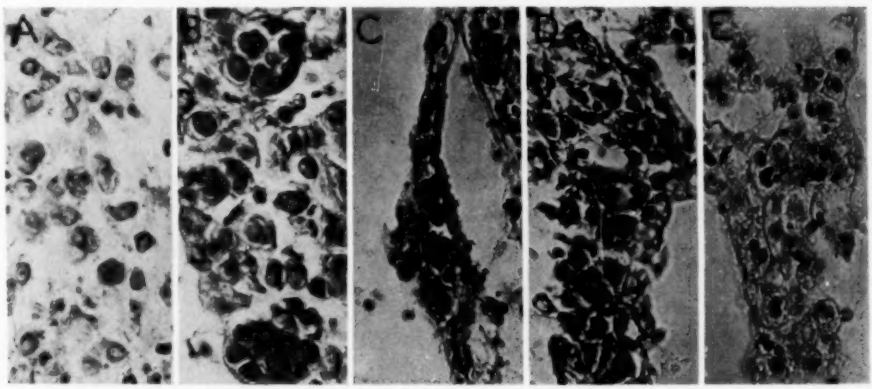

FIG. 8.—Membranes stained for cholinesterase taken under identical conditions (light filter, enlargement, developing and printing): *A*—24 hours after infection. *B*—32 hours after infection. *C*—2½ days after infection. *D*—3 days after infection. *E*—4 days after infection.

A CYTOCHEMICAL STUDY OF THE NATURE OF NEGRI BODIES

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The aim of the experiments reported below was to investigate whether rabies virus inclusion bodies undergo a developmental cycle similar to that found in herpetic inclusions,[1] i.e., whether the virus particles multiply in the inclusion up to a maximum, after which their number decreases.

MATERIALS AND METHODS

Three-week-old white mice were used. A different batch of four to six mice was inoculated every day intracerebrally with approximately 0.03 ml of the rabies virus suspension. The virus used was a street strain (Teheran I), kindly supplied by Professor P. Lepine of the Institut Pasteur, Paris, triturated and suspended in about 4 volumes of nutrient broth with the addition of 100 U. penicillin and 100 U. streptomycin to the total volume (approximately 3 ml).

On the eighth day, when some of the first inoculated mice died, the others were paralyzed and dying, and paralyses were observed in the next batch, the whole lot of mice was sacrificed. Thin slices of brain were fixed and treated in the same way as the chorioallantoic membranes described in the preceding paper,[1] i.e., after embedding, sections were stained with the hematoxylin and eosin, Giemsa and Feulgen's methods and treated for the demonstration of alkaline and acid phosphatase, lipase and cholinesterase.

RESULTS

In the sections of brains six hours after inoculation, no specific changes were observed. In those of one day, tiny basophilic granules were observed in the axons and axon hillocks of a few hippocampal cells. These granules, less than 1 μ in diameter, were Feulgen-positive (fig. 1). Similar granules, most probably identical in character, were stained heavily in the alkaline phosphatase and cholinesterase preparations. In sections of brains of the third to the fifth days after the inoculation, the number of inclusions was progressively greater and their distribution wider. While some inclusions were small and basophilic, others were rather large, contained one or more small basophilic bodies (Innenkoerper), surrounded by a clear or light eosinophilic matrix. The small inclusions and the Innenkoerper of the larger ones were Feulgen-positive. In some instances parts of the capsules of the larger bodies were also Feulgen-positive. All the Feulgen-positive structures gave a moderate to strong positive reaction for alkaline phosphatase and cholinesterase, and a weakly positive reaction for lipase. The lipase reaction, although rather weak, could be well observed, as it was usually stronger than that of all the surrounding cellular structures. The matrix of the larger inclusions gave in some places a positive reaction for alkaline phosphatase and cholinesterase, but the intensity of the reaction was much less than that of the Innenkoerper (fig. 2 and 3).

In the sections visualizing the acid phosphatase, the staining was patchy and irregular. In most sections the reaction was most intense in the areas which contained inclusions, and there

Received for publication December 14, 1951.

The authors are grateful to Prof. P. Lepine of the Institut Pasteur of Paris for the strain of rabies used in this experiment, to Dr. H. Bernkopf of the Laboratory for Virus Research of the Hebrew University for his help and advice, and to Mrs. H. Weinman for the microphotographic work.

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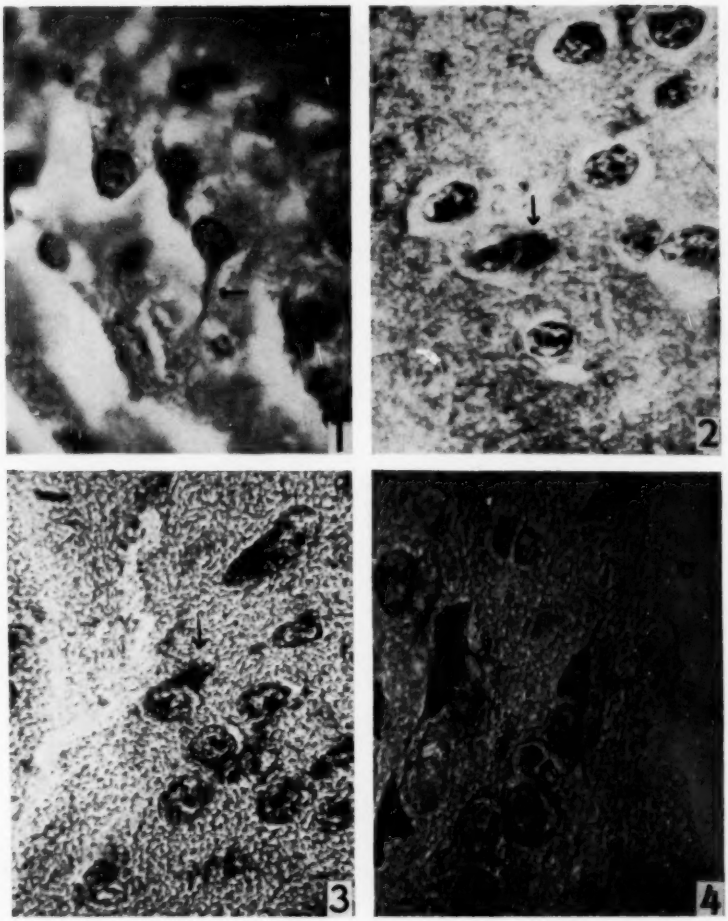

FIG. 1.—Nerve cell of a mouse sacrificed one day after infection. Feulgen-positive particles in axon and axon hillock. Feulgen-fast green. ×1000.

FIG. 2.—Negri body in an animal sacrificed four days after infection. Alkaline phosphatase. ×1000.

FIG. 3.—Negri body in an animal sacrificed four days after infection. Cholinesterase. ×1000.

FIG. 4.—Negri body in an animal sacrificed five days after infection. Acid phosphatase. ×850.

the inclusions gave a strong positive reaction (fig. 4). Around these and other cells in many such areas the intercellular substance gave a stronger positive reaction than any cellular constituents.

On the sixth day, the inclusions were numerous, and while most of them appeared as above, with Feulgen-positive Innenkoerper (positive also for alkaline phosphatase, lipase and cholinesterase), a few were amorphous, amphophilic or eosinophilic, and contained little or no Feulgen-positive matter.

On the seventh and eighth days, the

number of Feulgen-negative, eosinophilic bodies (so-called Lyssa bodies) increased, so that by the eighth day they predominated. These bodies gave a very slight to negative reaction with the alkaline phosphatase, cholinesterase and lipase techniques.

DISCUSSION

The evolution of the Negri bodies follows the same general pattern as that of the herpetic inclusions except for one essential difference, i.e., since rabies kills the experimental animal, the phase of quantitative decrease of virus material in the inclusion body is less conspicuous.

It is believed that the findings indicate that the virus of rabies multiplies within the nervous cells, and that the small, basophilic inclusions and the Innenkoerper of the larger inclusions represent aggregates of virus particles. The multiplication of the virus in the inclusion is indicated by an increase in size of the inclusion, or by an increase in size and in number of the Innenkoerper contained in the inclusion. The second phase, that of a progressive reduction in the number of virus particles, is manifested by the reduction of the intensity of basophilia of the inclusions, by the reduction and disappearance of Feulgen-positive matter in them, and by the reduced enzymatic activities of these bodies.

The different staining reactions of the Negri bodies, including the Feulgen reaction, in the hands of different observers[2] and of the same observer[3] may be explained by variations in "age" of the inclusions.

SUMMARY AND CONCLUSIONS

In white mice injected intracerebrally with street rabies virus the following consecutive changes were found:

Within one day of injection minute, basophilic, Feulgen-positive inclusions were noted. These inclusions subsequently grew in size, often became embedded in a Feulgen-negative matrix, and increased in number. From the sixth day on a progressively increasing proportion of inclusions became amorphous, eosinophilic and Feulgen-negative.

The Feulgen-positive inclusions were also positive for alkaline phosphatase and cholinesterase, and weakly positive for lipase. The decrease of these reactions paralleled the decrease in basophilia and Feulgen positivity.

The findings are considered to corroborate the assumption that inclusion bodies represent virus particles aggregates in which the virus particles multiply up to a certain limit, and then diminish in number.

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STUDIES ON THE TOXICITY OF TYPHUS RICKETTSIAE

I. SUSCEPTIBILITY OF THE WHITE RAT, WITH A NOTE ON PATHOLOGIC CHANGES

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The toxicity of typhus rickettsiae for white mice[1–3] is a phenomenon of practical as well as theoretical interest. The way in which rickettsiae cause the death of mice is not understood, but several important aspects of rickettsial toxicity have been clearly established. The toxic effects are intimately associated with the living rickettsial cells; there is close correlation between the toxicity for mice on the one hand and metabolic activity[4] or total number of viable microorganisms on the other hand.[5] Thus rickettsial suspensions can be rapidly and reproducibly titrated by observing the response of mice to intravenous inoculations. When the microorganisms are mixed with homologous antiserum, their toxicity is specifically neutralized, a fact which provides the basis for the standard neutralization test used in assaying the potency of typhus vaccines.[6] Furthermore, mice which have been previously infected or immunized resist challenge under appropriate conditions with toxic doses of the homologous organisms. Advantage of this has been taken in performing cross immunity tests.[3,7,8] Finally, the theoretical aspects of acute rickettsial toxicity are of considerable interest in comparison with viral and bacterial toxins. For these reasons the phenomenon has been under scrutiny in this laboratory from several viewpoints. Because our attempts to observe the physiologic responses of mice to the toxic properties of rickettsiae were severely handicapped by the difficulty in obtaining specimens in adequate amounts for analyses, a larger animal species was sought for studies of rickettsial toxicity.

Received for publication February 7, 1952.

This work was supported by a grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service, by a grant from the Lederle Laboratories Division of the American Cyanamid Company, and was conducted with the aid of the Commission on Virus and Rickettsial Diseases, Armed Forces Epidemiological Board, Office of The Surgeon General, United States Army, Washington, D. C.

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This paper reports the susceptibility of the white rat to the acute toxicity of typhus rickettsiae in a manner comparable to the phenomenon in white mice. A preliminary note is made of pathologic lesions associated with toxic death in rats.

METHODS

Rickettsial suspensions were prepared from pooled yolk sacs of infected embryonated eggs[9] and diluted in the buffer solution referred to as "sucrose PG."[10] Aliquots of yolk sac pools were stored in sealed glass ampoules which were thawed, then diluted, and maintained in an ice water bath until the moment of injection for each day's test. Several rickettsial strains were used: the Wilmington strain of murine typhus (*Rickettsia mooseri*), the Breinl strain of epidemic typhus (*Rickettsia prowazeki*), the Ankara strain[5] of epidemic typhus (isolated in 1943 in Turkey), the relatively avirulent epidemic strain E,[11] and the Cape Cod strain[6] of Rocky Mountain spotted fever (*Dermacentroxenus rickettsi*). The normal yolk sac suspensions for control inoculations were prepared from normal 14-day chick embryos and treated in the same manner as the infected pools. All yolk sac suspensions were sterile bacteriologically when tested on routine mediums.

Animals used throughout were white Swiss mice weighing 10 to 16 g and white male rats of the Hisaw Harvard Biological Laboratory strain, weighing 130 to 220 g. Both rats and mice were injected into the tail vein under light ether anesthesia after the tails were dipped briefly into warm water. Unless otherwise specified four animals were used for each dilution of inoculum, with a dose of 0.25 ml for mice and 1.0 ml for rats. Deaths were not attributed to the toxic properties of the rickettsiae if they occurred in less than 30 minutes or later than 24 hours after injection. The rickettsial suspensions were injected as half-logarithmic dilutions, such as 10^{-1}, $10^{-1.5}$, $10^{-2.0}$, etc., and LD_{50} of a given suspension was calculated by the method of Reed and Muench.[12]

Heat inactivation of rickettsial suspensions was carried out by complete immersion of a sealed glass ampoule below the surface of water at 56 to 57 C for 30 minutes. The suspension was then transferred to an ice water bath until injected. For the neutralization test a hyperimmune antityphus rabbit serum without preservative diluted 1:5 with sterile saline was mixed with an equal volume of the test rickettsial suspension and kept for one hour in an ice water bath before it was injected in a volume twice that of the usual dose. Normal rabbit serum constituted a neutralization control.

Rats surviving a sublethal toxic dose of epidemic rickettsiae were observed for at least 14 days for signs of infection. Observations on gross pathology were made usually within 30 minutes and never longer than two or three hours after death of the animal.

EXPERIMENTAL

Preliminary tests with rats were first done using weanling white rats, 5 to 6 weeks of age and weighing 40 to 70 g. The animals were injected into the tail vein with infected yolk sac suspensions of epidemic typhus (*R. prowazeki*). These preliminary experiments indicated that approximately 20 mouse LD_{50} toxic doses were sufficient to kill weanling white rats in less than 24 hours. It was next shown that adult white rats would also succumb to the toxic properties of the rickettsiae.

To establish the specificity of this phenomenon, several criteria were applied. The effect of intravenous injection of normal yolk sac suspensions was tested. It was found that adult rats tolerated 1 ml doses of up to $10^{-0.3}$, or 50% suspensions of normal yolk sac. Heating of infected yolk sac suspensions for 30 minutes at 56 C destroyed the toxic effect. Finally, it was demon-

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TABLE 1.—*Specificity of the reaction of white rats to the Breinl strain of epidemic typhus rickettsiae.*

Dose		Inoculum†	Number of rats	Weight in g	Results‡
Volume in ml	Dilution*				
1	0.3	Normal yolk sac	2	150	All survived
1	0.6	Normal yolk sac	3	110–170	All survived
2	1.3	Infected yolk sac§ in 1:10 normal rabbit serum	4	200–225	All died between 3 and 17 hours after injection
2	1.3	Infected yolk sac in 1:10 anti-typhus rabbit serum	4	200–230	All survived
1	1.0	Infected yolk sac	4	210–230	All died within 4 hours after injection
1	1.0	Infected yolk sac heated for 30 min. at 56 C	4	170–215	All survived

* Dilutions are expressed as the log of the denominator.
† Inoculum administered intravenously under light ether anesthesia.
‡ The animals were observed for 24 hours following inoculation.
§ Infected yolk sac suspension used throughout this experiment had an LD_{50} dose for rats of 1 ml of a $10^{-1.5}$ dilution.

strated that hyperimmune anti-epidemic typhus rabbit serum in a final dilution of 1:10 when mixed with the infected yolk sac suspension and kept in an ice water bath for one hour prior to injection protected against the lethal toxic effect. Normal rabbit serum under the same conditions failed to protect. These results are summarized in table 1.

Reproducibility of titration end points.—One feature of acute rickettsial toxicity in white mice which makes the test useful in the laboratory is the reproducibility of results under standard conditions with a given rickettsial suspension. If the phenomenon of acute toxicity in white rats is similar to that in mice, consistent results on repeated tests would be expected. Such evidence is shown in table 2 where the toxic effect of the same pool of epidemic typhus rickettsiae was titrated in both rats and mice on different days but always under standard conditions.

The values in table 2 indicate that the results in repeated titrations in white rats are reproducible, and that approximately 5 to 20 mouse LD_{50} doses are equivalent to one rat LD_{50} dose of the Breinl strain of rickettsiae. Since the rats weighed roughly ten to 15 times as much as the mice, it seems that on a weight basis the LD_{50} of the Breinl strain of epidemic rickettsiae is nearly identical for the white rats and white mice used in our tests.

Response of white rats to different rickettsial strains.—It was considered essential to observe the effects of other rickettsial strains in white rats in order to evaluate the results obtained above with the Breinl strain. In table 3 the data are presented from titrations using (a) an epidemic strain originally iso-

TABLE 2.—*Repeated titrations of same rickettsial suspension (Breinl strain of* R. prowazeki*) for acute toxicity in white rats and white mice.*

Date of experiment	Test animal	Weight in g	Dose in ml	Survivors at 24 hours* Total inoculated per group				LD_{50}*†
				1.0	1.5	2.0	2.5	
6/19/51	Rat	130–160	1.0	0/4	1/4	4/4		1.67
	Mouse	10–15	0.25	0/3	0/3	3/4	4/4	1.88
6/22/51	Rat	140–175	1.0	0/4	0/4	4/4		1.75
	Mouse	10–15	0.25		0/4	3/4	4/4	1.88
6/29/51	Rat	150–230	1.0	0/4	2/4	4/4		1.50
	Mouse	10–16	0.25		0/4	1/4	4/4	2.16

* Dilutions expressed as the log of the denominator.
† Calculated according to the method of Reed and Muench.[12]

lated in Ankara, Turkey, (b) the relatively avirulent epidemic strain E, (c) the Wilmington murine strain, and (d) the Cape Cod strain of Rocky Mountain spotted fever. The LD_{50} doses for white rats of the Ankara and E strains were equivalent to approximately 10 LD_{50} doses for white mice, and thus were very similar for all three epidemic strains.

With murine typhus, however, a different relationship was noted. Preliminary tests with murine typhus rickettsiae in weanling rats suggested that the rat was more resistant to the acute toxic effect of murine rickettsiae than to those of epidemic typhus strains. These observations were supported by a subsequent experiment in which more than 100 mouse LD_{50} doses were required to kill adult white rats.

The only other rickettsial agent tested for acute toxicity in adult white rats was the Cape Cod strain of Rocky Mountain spotted fever. A yolk sac suspension with an infectivity titer of 10^{-5} in embryonated eggs failed to show either lethal toxicity or evidence of subsequent infection in white rats injected intravenously with 1.5 cc of $10^{-0.6}$ dilution of infected yolk sac. White mice were also unaffected by the same spotted fever strain. Rickettsiae of scrub typhus (*Rickettsia tsutsugamushi*) were not tested for their acute toxic effect in white rats although the Gilliam strain is known to be toxic for mice.[13]

Manifestations of toxicity.—Significant manifestations of acute rickettsial toxicity in white rats with the dosages employed were not apparent until about 30 minutes or more after injection. The more prominent features included huddling together of the animals, labored respiration, cyanosis, and finally prostration. Times of death after injection varied from 1½ to 4 hours with larger doses, and up to 18 hours with doses nearer the LD_{50} range. The manifestations of acute toxicity observed in rats thus are similar to those described as occurring in mice, with the exception that hyperirritability and convulsive movements were not noted in rats.

Pathologic findings.—Rats dying of rickettsial toxicity were autopsied within a few minutes to several hours after death. The only constant and significant gross findings were confined to the small intestine. The visceral blood vessels in general, and those of the small intestine in particular, were enlarged and congested with blood. The lumen of the gut often contained fluid

TABLE 3.—*Acute toxicity of different rickettsial strains in white rats as compared with white mice.*

Rickettsial strain	Test animal	Weight in g	Dose in ml	Survivors at 24 hours: Total inoculated per group at dilutions*								LD_{50}*,†
				0.6	0.7	1.0	1.1	1.5	1.6	2.0	2.5	
Epidemic Strain E	Rat	130–180	1.0			0/4		0/4		4/4		1.75
	Mouse	10–16	0.25					0/4		1/4	4/4	2.16
Epidemic Turkish Ankara strain	Rat	150–190	1.0	0/3			1/4		4/4			1.26
	Mouse	10–16	0.25				0/4		2/4	4/4		1.60
Murine Wilmington strain	Rat	180–230	1.0		2/3	2/2						>0.07
	Mouse	10–16	0.25					0/4		0/4	4/4	2.25

* Dilutions expressed as the log of the denominator.
† Calculated according to the method of Reed and Muench.[12]

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which was yellowish in color and sometimes frankly bloody. These changes in the small intestine varied in degree from one animal to another but were quite constant with larger toxic doses when death resulted within three to four hours after injection. With such doses the mid portion of the small bowel was usually a dark reddish color and often appeared hemorrhagic.

After these findings in rats had been noted, mice succumbing to toxin were examined more closely; the same visceral congestion was observed, with the small intestine most prominently affected although the changes were not nearly as prominent as those observed in the rats. Only occasionally in mice did the gut appear to be hemorrhagic. The mucosa of the small bowel, however, was often covered with a yellowish fluid and the blood vessels of the villi were markedly dilated. These patholgoic findings in toxic rats and mice are being studied further in association with histologic studies.[14]

The possibility was considered that the gross lesions of the small bowel in rats might be related in some way to venous portal drainage from the route of tail vein injection. This hypothesis was tested by injecting four rats into the heart and two rats into a tail vein in the usual manner with a known toxic dose. Upon autopsy immediately after toxic death two of the four rats injected intracardially showed prominent gross small bowel lesions approximately equal in degree to those seen in the two intravenously injected rats. The other two intracardially injected rats showed only minimal gross small bowel changes. This experiment was interpreted as evidence that the site of inoculation into the vascular bed probably does not determine the location of the main pathologic changes.

Response of white rats to sublethal toxic doses.—Adult white rats which survived sublethal toxic doses were observed two weeks for infection deaths. Rats receiving epidemic typhus strains showed no signs of typhus infection during this time. However, serologic evidence of a complement-fixing antibody response to a single intravenous sublethal dose was obtained. On the other hand, all rats which were injected with murine rickettsiae and which survived the acute toxicity subsequently died of infection in the period from 48 to 72 hours after injection.

DISCUSSION

On the basis of the criteria applied in this study it has been shown that the white rat is susceptible to the acutely lethal action of typhus rickettsiae. The essential features of this toxic phenomenon appear to be identical in rats and mice. The larger size of the white rat is an obvious advantage for studies on certain aspects of the mechanism of acute rickettsial toxicity.

It should be remembered that relatively enormous numbers of viable rickettsiae are required to produce the acute lethal effect in either mice or rats. Consequently, the technical difficulties attendant upon the concentration of viable rickettsiae seriously limit attempts to demonstrate acute rickettsial toxicity in larger laboratory animals. Such considerations may explain in part the failure of rickettsial strains of the spotted fever group and most scrub typhus strains to elicit acute rickettsial toxicity in mice. However, other factors besides numbers of rickettsiae are probably involved. Smadel and associates[15] injected white rats intravenously

14. Parker, F., Jr. and Neva, F. A. Unpublished observations.

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with rickettsiae of scrub typhus in doses up to 2 ml of 10% yolk sac suspensions which had infectivity titers of 10^{-8}. Their experiments were designed to produce overwhelming infection for studies of antigen in tissues and body fluids, but they did not report deaths in the rats before four days. However, the strains of scrub typhus used for this work did not include the Gilliam strain, which was subsequently found to exhibit acute toxicity in mice.[13] Although cotton rats injected intracardially with 25 to 50 toxic doses for mice succumb to infection as early as three days after inoculation, it has not been possible to demonstrate their susceptibility to rickettsial toxic effect.[5] Our results in the present report with murine typhus rickettsiae in rats are additional evidence that the rickettsial strain or animal species used in the test may be important factors in the phenomenon of acute rickettsial toxicity.

The prostration and labored respirations of rats and mice succumbing to acute rickettsial toxicity have similarities to shock. A description of altered physiology associated with rickettsial toxicity was presented by Clarke and Fox,[16] who noted hemoconcentration and apparent reduction in circulating blood volume in toxic mice. Studies in this laboratory[17] have confirmed and extended their findings.

Burrows, in a recent review[18] of bacterial endotoxins, points out that hemorrhage of the small intestine is frequently found in animals injected with a fatal dose of various bacterial endotoxins. He also comments on the similarity of circulatory changes in animals injected with bacterial endotoxins to circulatory changes characteristic of traumatic shock. It is of interest to recall that in studies of toxicity of influenza viruses for mice,[19] although liver lesions were the most prominent, it was noted that animals injected with some strains of influenza A virus were described as sometimes showing vascular engorgement, edema, and bloody mucous material in the intestine. Like the toxicity of typhus rickettsiae, the toxicity of influenza viruses[19] and viruses of the lymphogranuloma-psittacosis group[20] seems to be dependent upon or associated with the living microorganisms. Whether any similarity exists in the mechanisms of toxic phenomena produced by material derived from dead bacteria in one instance and viable rickettsiae or viruses in the other instance can only be a matter of speculation at the present time.

After the studies now reported had been completed, a report was found in the German literature by Siegert[21] dealing with microscopic changes attributed to acute rickettsial toxicity, in which he refers to the susceptibility of white rats to rickettsial toxin. However,

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Soluble antigen in tissues and body fluids of infected mice and rats. J. Exper. Med. **83**: 133–146.

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20. Rake, G. and Jones, H. P. 1944, Studies on lymphogranuloma venereum. II. The association of specific toxins with agents of the lymphogranuloma-psittacosis group. J. Exper. Med. **79**: 463–486.
21. Siegert, R. 1948, Das Rickettsientoxin als Ursache histopathologischer Organveränderungen. Ztschr. f. Hyg. u. Infektionskr. **128**: 477–485.

Siegert's article presented no data on neutralization of toxicity with specific antiserum, dosages of rickettsial suspensions required, relationship of the toxicity to viability of the rickettsiae, or behavior of various rickettsial strains when tested for acute toxicity. In addition, Siegert made no mention of gross pathologic lesions in the small bowel in his discussion of the pathology of acute rickettsial toxicity in the white rat.

SUMMARY

Susceptibility of the white rat to the toxic properties of intravenously administered living typhus rickettsiae is reported. Evidence is presented which shows that the toxicity is due specifically to living typhus rickettsiae and is similar to the phenomenon in white mice.

In repeated titrations of the toxic dose for rats as compared with mice, it was shown that the white rat is as susceptible as the mouse on a body weight basis to acute toxicity with epidemic typhus rickettsiae, but more resistant than the mouse to toxicity with rickettsiae of murine typhus.

Gross pathologic changes were observed in the small intestine of white rats dying of acute rickettsial toxicity.

IN VITRO AND IN VIVO ACTION OF AUREOMYCIN ON VACCINIA VIRUS IN THE CHICK EMBRYO AND IN THE RABBIT

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Vaccinia virus has generally been found to be unaffected by chemotherapeutic agents. Of 74 different compounds tested in vitro by Andrewes and others,[1] none was of any value. Several other investigators have reported that sulfonamides had no effect on vaccinial infections.[2–5] Thompson,[6] however, studied a number of metabolites, metabolite antagonists and enzyme inhibitors in virus-infected tissue cultures and found some of these compounds to have an inhibitory action on the multiplication of vaccinia virus.

Most workers[1,6–9] found penicillin to have no effect on the virus in vitro or in vivo (in man, animals, tissue cultures or eggs). Commercial but not pure penicillin showed some antibiotic effect on vaccinia virus in eggs in the hands of Groupé and Rake.[10] Streptomycin also was proved to be of no value against vaccinia virus.[8]

More recently, Perry and Martineau[11] treated a child suffering from eczema vaccinatum with aureomycin and reported the prompt recovery of the patient. They thought that aureomycin acted either through indirect control of secondary bacterial infection or through some specific antibiotic action on the virus. Conversely, Baldridge and Kligman in a preliminary report[12] failed to demonstrate in the infected chick embryo any inhibiting activity against vaccinia virus by aureomycin or terramycin. In addition, Marsden and Coughlan,[13] Breen[14] and Stolte and Sas[15] found no indication that smallpox virus was affected by aureomycin, terramycin or chloramphenicol in human cases of the disease.

The object of this paper is to present data on the in vitro and in vivo action of aureomycin on vaccinia virus, using fertile hens' eggs and rabbits as experimental hosts.

Received for publication February 21, 1952.

The authors express their appreciation to Mrs. E. Smalley for her valuable technical assistance and to Mrs. A. K. Lamb for her suggestions in the preparation of the manuscript.

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MATERIALS AND METHODS

Strain of virus.—The vaccinia virus used in these experiments was an egg-adapted strain of calf lymph virus in its fifth egg passage. The parent strain had originally been isolated from scabs of human vaccinations in 1909, and has since been used for the commercial production of calf lymph vaccine.

Egg inoculations

1. *Virus.*—Virus inoculations were made on the chorioallantoic membrane (C.A.M.) or in the allantoic sac (A.S.) of 11-day-old chick embryos. Virus dilutions were made in sterile buffered water at pH 7.0 to 7.2 and an 0.25 ml dose of inoculum was always used. To insure bacterial sterility, enough penicillin and streptomycin were added to the virus suspension before inoculation to obtain a final concentration of 500 units and 100 μg of each per ml, respectively. As a greater concentration of vaccinia virus is required to produce lesions and death when the A.S. route is used as compared with C.A.M. inoculation, the following dilutions of virus were always used: 10^{-3} for A.S. inoculation and 10^{-5} for C.A.M. inoculation.

C.A.M. inoculations.—In the in vitro contact experiments vaccinia virus was first diluted to 10^{-3} in a suspension of aureomycin containing 8 mg of the antibiotic per ml. After the desired time of incubation, the virus mixture was further diluted to 10^{-5} and then injected on the C.A.M. In the in vivo experiments, the virus was diluted to 10^{-5} and deposited on the C.A.M. without further dilution; aureomycin was subsequently injected into the A.S.

Eggs receiving 0.25 ml of the untreated inoculum and incubated at 36 C were generally found dead in 96 to 120 hours. Occasionally they survived up to 192 hours. All infected embryos were hemorrhagic and the portion of the C.A.M. at the base of the artificial air sac was thickened and covered with individual or confluent pock lesions typical of vaccinia.

A.S. inoculations.—In the in vitro contact experiments vaccinia virus was diluted to 10^{-3} in a suspension of aureomycin containing 8 mg per ml. After the desired incubation period, the suspension was injected into the A.S. without further dilution. In the in vivo experiments the 10^{-3} virus dilution was directly inoculated into the A.S., and aureomycin was subsequently injected by the same route.

Embryos thus infected were again found dead in 96 to 120 hours, and pocks on the C.A.M. were generally scattered throughout the membrane, although they were less numerous and showed practically no confluency as compared with the C.A.M. inoculations. Frequently, however, a large confluent lesion could be observed on the C.A.M. at the point of inoculation.

Interpretation of results.—Embryos found dead 24 to 48 hours following virus inoculation and whose death could not be associated with virus multiplication by the presence of pocks on the C.A.M. were considered to have died of trauma or other nonspecific causes. These are listed in the tables as "embryos prematurely dead." Those which died after 48 hours were examined and their membranes were checked for typical lesions. Embryos living at 192 hours were also examined for presence or absence of lesions.

2. *Aureomycin.*—Crystalline aureomycin hydrochloride was resuspended to the desired concentration with sterile buffered water at pH 7.0 to 7.2 just before injection into eggs or mixing with vaccinia virus. In some instances aureomycin suspensions or mixtures were deposited on the C.A.M., but in most cases they were injected into the A.S.* In two experiments, aureomycin was inactivated at 56 C for 43 hours. The assays of two lots thus treated decreased from 6400 to 10.8 μg per ml and from 7850 to 37 μg per ml, respectively. The pH of the inactivated lots of aureomycin varied between 6.8 and 6.5 during the course of the experiments.

In all cases 2 mg active or inactivated aureomycin were injected into each egg either once or repeatedly.

Rabbit inoculations

1. *Virus.*—The rabbits were obtained from local dealers, were generally light or medium gray in color, and weighed six to eight pounds. The flanks were clipped and virus was titrated by a single scratch method as follows: Each test sample and a known control virus preparation were diluted at 1:1000, 1:3000, 1:10,000, 1:30,000 and 1:100,000 in beef heart infusion broth. For each titration, five superficial scratches of about 5 cm in length were made on the shaved flanks of the rabbit with a sharp needle. Each dilution was then rubbed vigorously into one scratch with the help of the blunt end of a glass tube. The excess fluid was then removed to prevent spread. Inoculated rabbits were kept in individual cages for five

* The toxicity of the antibiotic for the chick embryo was determined by means of a titration in eggs by the A.S. route with the following results: 5 mg per egg killed 4 out of 5 embryos in 4 days; 4 mg killed 5 out of 6 embryos in 4 days; 3 mg killed 3 out of 6 in 5 days. When 2 mg aureomycin were injected, 5 out of 6 embryos survived beyond the 10th day following injection.

days, at which time the length of the scratch and the extent of the lesion were measured in millimeters and the percentage, or "take," calculated. The mean average of the five dilutions was compared with that of the control virus.

2. *Aureomycin.*—Aureomycin was either mixed in vitro with the virus suspension before titration on the rabbit or was administered intravenously after the animal was infected.

EXPERIMENTAL

Experiments in eggs

In the initial experiments, aureomycin suspensions or virus-aureomycin mixtures were inoculated on the C.A.M. In later experiments the methods of inoculation described below were used in order to avoid the toxic effect of aureomycin when injected on the C.A.M.

1. *In vitro contact experiments.*—A 10^{-3} virus dilution was made in an aureomycin suspension containing 8 mg antibiotic per ml. Samples of the mixture were taken after one and two hours incubation at 35 C and after 16 to 18 hours at room temperature (24 C). Immediately after each sample was taken, it was injected in the A.S. of 11-day-old eggs. The remainder of each sample was then diluted to 10^{-5} and deposited on the C.A.M. of another lot of eggs. The inoculated eggs were sealed and returned to the incubator. Results of this experiment are summarized in table 1. It may be seen that aureomycin had a definite inhibitory action upon the multiplication of the virus by the A.S. route. This action was in direct proportion to the length of the incubation period of the virus-aureomycin mixture. However, after dilution to 10^{-5} and inoculation on the C.A.M., aureomycin failed to show any such action on the multiplication of vaccinia virus. In order to determine whether the inhibiting action of aureomycin in the A.S. was

TABLE 1.—*In vitro action of aureomycin on vaccinia virus in the developing chick embryo.*

Virus dilution injected	Incubation period of aureomycin-virus mixture* prior to inoculation				Site of injection	Embryos prematurely dead	Test embryos dead of infection		Control embryos dead of infection	
	Active aureomycin		Inactive aureomycin				Number	Percent	Number	Percent
	Time (hrs.)	Temp. (Cent.)	Time (hrs.)	Temp. (Cent.)						
10^{-3}	1	35			A.S.	1	5/8†	62	10/10	100
	2	35				3	12/34	35	29/29	100
	16–18	24				0	0/10	0	10/10	100
10^{-3}			1	35	A.S.	1	0/8	0	10/10	100
			2	35		1	0/17	0	19/19	100
10^{-5}	1	35			C.A.M.	1	19/19	100	18/20	90
	2	35				0	21/27	78	31/39	83
	16–18	24				0	10/11	90	9/10	90
10^{-5}			1	35	C.A.M.	0	1/9	11	18/20	90
			2	35		2	1/16	6	31/39	83

* The original virus dilutions of 10^{-3} were made in an aureomycin suspension containing 8 mg antibiotic per ml.
† Five embryos died of infection out of 8 surviving beyond 48 hours.

at the rate of 2 mg of the antibiotic per egg. Most embryos receiving the antibiotic by this route died prematurely, presenting no characteristic vaccinia lesions and showing a heavy yellow precipitate on the C.A.M. It seemed that aureomycin, which had been proved to be nontoxic to the embryo at that level when administered in the A.S., had a deleterious effect when concentrated on a single portion of the C.A.M. at the base of the artificial air sac. However, the few embryos that survived beyond 48 hours subsequently died at 120 or 144 hours and exhibited well defined vaccinia pocks, thus showing that aureomycin had no effect on the virus.

specific for the active principle of the antibiotic, or was due to a nonspecific factor, another in vitro experiment was set up as follows: An aureomycin suspension with a final pH of 6.7 containing 8 mg per ml was made in buffered water. The suspension was inactivated by heating in a waterbath at 56 C for 43 hours, after which it was used to make a 10^{-3} dilution of vaccinia virus. Samples of the mixture incubated at 35 C were removed at one and two hours, respectively, and immediately seems that an unknown, nonspecific, heat stable factor is responsible for its in vitro action on vaccinia virus.

2. *Simultaneous in vitro and in vivo contact experiments.*—In an attempt to determine the required in vitro contact period for aureomycin to act on vaccinia virus, as observed in experiment 1, the following series of inoculations were made (see table 2): Eight lots of eggs were used, and in each the final dose of aureomycin per egg was 2 mg and the dilution of virus was constant for the

TABLE 2.—*Action of aureomycin on vaccinia virus: administration of aureomycin at varying intervals before or after inoculation of virus.*

Egg lot Num-	Site of injection*		Administration of aureomycin (hrs.)	Embryos prematurely dead	Test embryos	Control embryos
	Virus	Aureomycin			Number dead of infection	Number dead of infection
1†	C.A.M.	A.S.	−24	0	9/9§	
2	C.A.M.	A.S.	Immediately after virus inoculation	3	7/7	10/10
3	C.A.M.	A.S.	+24	1	9/9	
4‡	A.S.	A.S.	−24	0	9/9	
5	A.S.	A.S.	Immediately after virus inoculation	0	10/10	
6	A.S.		Immediately after mixture with virus in vitro	1	6/9	10/10
7	A.S.		+2 after virus-aureomycin incubated at 35 C	0	1/10	
8	A.S.	A.S.	+24	1	9/9	

* Each embryo received 2 mg aureomycin
† Lot numbers 1, 2 and 3 received a 10^{-3} virus dilution.
‡ Lot numbers 4 to 8 received a 10^{-2} virus dilution.
§ Nine embryos died of infection out of 9 surviving beyond 48 hours.

injected into the A.S. of embryonated eggs. The remainder of the samples was further diluted to 10^{-5} and deposited on the C.A.M. of another lot of eggs. The results of this experiment are also summarized in table 1.

It may be observed that the inactivated aureomycin, when injected into the A.S., had a more pronounced effect on vaccinia virus than active aureomycin, and that it completely inhibited the growth of the virus. Furthermore, unlike the experiment in which active aureomycin was used, the majority of embryos inoculated on the C.A.M. with the 10^{-5} dilution survived with no indication of virus multiplication. This effect of inactivated aureomycin is not understood at the present time, but it route of inoculation. One set of tests was made by C.A.M. inoculation of the virus and a parallel simultaneous series by injection into the A.S. In one pair of tests the antibiotic was introduced into the A.S. 24 hours prior to virus exposure (lots 1 and 4); in a second set the injections were made immediately one after the other (lots 2 and 5); and in the third the antibiotic was introduced 24 hours after virus inoculation (lots 3 and 8). In addition, two groups of eggs were inoculated by the A.S. route with a combination of virus and antibiotic premixed in vitro; one group received the combination immediately after mixing (lot 6) and the other after two hours contact at 35 C (lot 7). The results of this series of inoculations are given in

table 2. It may be noted that when the virus was inoculated on the C.A.M., aureomycin administered 24 hours before, simultaneously with, or 24 hours after the virus had no effect on its multiplication. When the A.S. route was chosen for virus inoculation, aureomycin again failed to affect the virus when injected 24 hours before, simultaneously with, or 24 hours after the virus. However, an in vitro contact of virus with aureomycin for as short a period as a few minutes was sufficient to cause a decrease in the infectivity rate to 66%. A more prolonged contact period of two hours at 35 C brought the infectivity rate down still further to 10%.

3. *In vivo contact experiments.*—A 10^{-3} virus dilution in buffered water was made and immediately inoculated into one lot of eggs (no. 1) via the A.S. A 10^{-5} dilution of the same virus was also made and inoculated into two lots (2 and 3) of eggs on the C.A.M. All three lots of embryos were returned to the incubator for 24 hours. Eggs of lot 1 were then each injected in the A.S. with 0.25 ml of aureomycin suspension containing 2 mg of the antibiotic and returned to the incubator. The same operation was repeated three more times at 24-hour intervals, for a total dosage of 8 mg of antibiotic.

Eggs of lot 2 received a single injection of aureomycin at the end of 24 hours incubation, while eggs of lot 3 received two such injections at 24 and 48 hours following inoculation of virus. In each of these instances aureomycin was administered into the A.S. in 2 mg amounts per egg and in 0.25 ml volume.

The results of these experiments have been summarized in table 3. It can be seen that whatever the route of inoculation or the frequency of administration of active aureomcyin, the antibiotic failed to show any deleterious action upon the multiplication of vaccinia virus.

In order to determine whether inactivated aureomycin exerted the same action by in vivo contact as by in vitro contact, the following inoculations were made: Two lots of eggs (1 and 2) were inoculated in the A.S. with a 10^{-3} dilution of virus, and two more lots (3 and 4) received a 10^{-5} dilution of the same virus on the C.A.M. After 24 hours, lot 1 and 3 received three injections of 2 mg active aureomycin at 24-hour intervals into the A.S. Lot 2 and 4 received inactivated aureomycin under similar conditions. Results of this experiment are summarized in table 4. Inactivated as well as active aureomycin failed to show any inhibiting in vivo action on the development of vaccinia virus.

TABLE 3.—*In vivo action of aureomycin on vaccinia virus in the developing chick embryo.*

Egg lot no.	Aureomycin*	Site of virus injection†	Administration of aureomycin: hours after virus injection				Embryos prematurely dead	Test embryos	Control embryos
			24	48	72	96		Number dead of infection	Number dead of infection
1		A.S.	X	X	X	X	2	35/35‡	10/10
2		C.A.M.	X				6	9/9	6/6
3		C.A.M.	X	X			8	17/17	
1	Active	A.S.	X	X	X		1	11/11	11/11
2		A.S.	X	X	X		0	12/12	
3	Inactive	C.A.M.	X	X	X		1	11/11	12/12
4		C.A.M.	X	X	X		1	11/11	

* Aureomycin was injected in 2 mg doses in the A.S. in this series of experiments.
† 10^{-3} into A.S. and 10^{-5} on C.A.M.
‡ Thirty-five embryos died of infection out of 35 surviving beyond 48 hours.

TABLE 4.—*In vitro activity of aureomycin on vaccinia virus, as demonstrated by the single scratch rabbit test.*

Rabbit no.	Incubation of aureomycin-virus mixture		Mean "take" values (percent)		Ratio
	Time (hrs)	Temp. (Cent)	Aureomycin-virus mixtures	Virus control suspension	
242	1	24*	86.4	86.8	0.98
243	1	24	77.8	72.8	1.06
246	16–18†	4	67.0	86.4	0.77
247	16–18	4	71.2	80.6	0.88
248	1	24	67.8	71.4	0.94
249	1	24	90.8	86.0	1.05

* Room temperature.
† Overnight.

Experiments in rabbits

1. *In vitro experiments.*—In the first set of experiments, each of the virus dilutions, described under *Methods*, was divided into two parts: to one part 8 mg crystalline aureomycin per ml were added, while the other served as control. After one hour incubation at room temperature (24 C), both series of dilutions were inoculated into rabbits 242 and 243, as given under *Methods*. The remainder of both series of dilutions were then held 16 to 18 hours at 4 C and inoculated in a similar manner into rabbits 246 and 247.

In the second set of experiments, a 1:100 virus dilution was made in an aureomycin solution containing 10 mg of the antibiotic per ml. After standing at 24 C for one hour this virus-aureomycin mixture was used to make the same series of dilutions as described above. A control series of virus dilutions, not containing aureomycin, was prepared in an identical manner. Both sets of dilutions were then used to inoculate rabbits 248 and 249. The results of the two sets of experiments have been summarized in table 5. One hour incubation of the virus-aureomycin mixture at room temperature apparently exerted no appreciable action on vaccinia virus. With the mixture held 16 to 18 hours at 4 C a slight drop in the mean take value was observed on both rabbits with the virus-aureomycin mixture, as compared with the control. However, this drop does not seem to be very significant because of the variations known to occur in such tests with the same virus preparation.

TABLE 5.—*In vivo activity of aureomycin on vaccinia virus, as demonstrated by the single scratch rabbit test.*

Rabbit no.	Daily dosage of aureomycin mg/1 kg	Administration of aureomycin		Mean "take" values (percent)	Average "take" values (percent)
		Route	Duration (days)		
242A	None			87.4	73.8
243A	None			60.2	
244	10	I.V.*	10	75.2	78.2
245	10	I.V.	10	81.2	

* Intravenous.

2. *In vivo experiments.*—Rabbits 242A, 243A, 244 and 245 were inoculated by the single scratch method with a set of virus dilutions from 1:1000 to 1:100,000. Beginning the next day and daily thereafter for ten days, rabbits 244 and 245 were injected intravenously with 10 mg aureomycin per kg of body weight. Rabbits 242A and 243A were left untreated to serve as controls. Measurements of the dermal lesions were made at five, six and seven days and mean take values calculated. Table 5 shows no difference in the average take values between treated and untreated rabbits. Furthermore, no appreciable difference was observed in the forming and falling of the scabs between treated and untreated rabbits.

DISCUSSION

It has been shown in the experiments described above that aureomycin per se does not have any in vivo action on the multiplication of vaccinia virus in fertile hens' eggs or in rabbits. This is in contrast to some clinical reports in the literature. It seems that if aureomycin has any effect on the course of the clinical disease, it may be the result of either

the controlling of secondary infection by aureomycin or of the action on the virus of some nonspecific "impurity." For, in the experiments described above, definite virucidal action in vitro was observed with active aureomycin, and that action was even greater after the active component of aureomycin was destroyed by heat. No such action, however, was observed when the inactivated aureomycin was used for the in vivo treatment of infected chick embryos.

A similar in vitro inhibiting action was reported by Groupé and Rake[10] with inactivated commercial penicillin on canary pox, fowl pox and vaccinia viruses, although crystalline penicillin had no action whatever on the multiplication of these viruses.

SUMMARY

In a series of in vitro and in vivo experiments in hens' eggs and rabbits, aureomycin was found to have no appreciable action on the development of vaccinia virus. Some inhibiting action was observed in the chick embryo when aureomycin and virus were mixed in vitro before inoculation. However, this inhibition was apparently caused by an unknown, nonspecific, heat stable factor.

STUDIES ON THE IMMUNOGENIC PROPERTIES OF *BACTERIUM TULARENSE* VARIANTS

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The detection of colony type variants in cultures of *Bacterium tularense* and a study of certain properties of these variants were recently reported by Eigelsbach, Braun, and Herring.[1] The majority of stock cultures examined were heterogeneous in that they contained cells which developed into colonies with distinct morphological characteristics detectable when a transparent solid medium and oblique lighting were used. New colonial types were also found following growth of originally homogeneous populations in liquid mediums. Pure cultures of almost all colony types could be maintained on solid mediums if frequent transfers were avoided. The colonial types could be classified on the basis of appearance, but a more useful characteristic of classification was the acriflavine reaction[2] produced by cells of certain colony types. On basis of this reaction, the various types were designated as either smooth-reacting or nonsmooth-reacting, and it was demonstrated that a variety of stable colonial types could be assigned to either of these two groups. Of considerable significance was the finding that high virulence was associated only with certain smooth-reacting types. All nonsmooth-reacting variants displayed significantly less virulence. In addition, preliminary data indicated that immunogenic properties of the respective variants are associated with certain smooth-reacting types.

In view of reports on the use of live vaccines in man or animals by Gotschlich,[3] Vereninova et al,[4] Kosmachevsky,[5] Downs et al,[6] and Downs and Woodward,[7] it became of interest to determine in more detail the immunogenic properties of the components of a particular culture of *Bact. tularense* which has been used for extensive vaccine studies. This culture is designated Jap and, according to Downs and Woodward,[7] is capable of protecting mice against 1000 LD_{50} of two highly virulent strains of *Bact. tularense*. Strain Jap is considered to be only relatively low in virulence. Inspection of the stock culture of this strain, as previously reported by Eigelsbach et al,[1] revealed the presence of two smooth-reacting colony types. Upon aging in

Received for publication March 6, 1952.

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broth for five days, an additional smooth-reacting and a nonsmooth-reacting type were recovered. These variants, as well as the heterogeneous parent culture, were used in the detailed analysis of immunogenic properties to be reported here. In addition, a nonsmooth-reacting, relatively avirulent variant obtained from the highly virulent strain Schu was used for these studies.

MATERIALS AND METHODS

The cultures used for vaccine production included (a) the heterogeneous Jap culture which contained primarily the Jap S2 type and a small proportion of Jap S1, both variants being smooth-reacting but differing in their colony morphology on peptone cysteine agar;[1] (b) Jap S1 isolated from Jap; (c) Jap S2 isolated from Jap; (d) a smooth-reacting colony type designated Jap S3 that was originally isolated following five days of growth of the Jap culture in peptone cysteine broth; (e) a nonsmooth-reacting colony type designated Jap S4^{ns}* also isolated from a five-day culture of Jap; and (f) a nonsmooth-reacting, rough appearing variant designated Schu NS3 obtained from a four-day liquid culture of the highly virulent Schu strain. These types proved to be stable during maintenance on glucose cysteine blood agar (GCBA) at 5 C when transfers were made at monthly intervals.

Live vaccines were prepared by suspending in 0.1% gelatin saline the growth obtained from GCBA slants which had been incubated at 37 C for 24 hours. The suspensions were adjusted turbidimetrically and the concentration of viable cells was verified by plate counts on GCBA.

White mice weighing 20 to 22 g or rats weighing 115 to 145 g were vaccinated by administering subcutaneously a single dose of approximately 10,000 organisms per animal. In each test, one group of animals was injected with *Bacillus globigii* according to the same procedure in order to detect possible production of nonspecific immunity. Fourteen days after vaccination all mice were tested for immunity subcutaneously or intraperitoneally with graded tenfold dilutions of a highly virulent clone, Schu S1 of *Bact. tularense;* vaccinated rats were similarly challenged except that graded 100-fold dilutions were employed. The heart's blood and spleen of animals dying after inoculation were cultured on GCBA.

In the experiments in which the rate of multiplication of variants within hosts was determined, five mice and five rats of each group were sacrificed at intervals of 2, 6, 10, and 14 days after vaccination. Their spleens were macerated and the number of viable cells of *Bact. tularense* per gram of spleen was determined by cultural methods, employing 0.5 ml of a 1:10 dilution of spleen homogenate. Weight and size of spleens were recorded.

* The acriflavine reaction is indicated as an exponent whenever it fails to correlate with colony appearance.

TABLE 1.—*Recovery of organisms from spleens and spleen weights of mice inoculated subcutaneously with live variants of* Bact. tularense.

Strain or colony type used as live vaccine	Intraperitoneal mouse LD_{50} (organisms)	Organism per g spleen; Days after vaccination				Average weight of spleen in mg; Days after vaccination	
		2	6	10	14	10	14
Jap (parent)	320,000	4,320	3,565,000	2108	210	332	370
Jap S1	320,000,000	0	0	0	0	154	108
Jap S2	320,000	333	2,564,100	1110	342	450	292
Jap S3	63,000,000	0	0	0	0	100	90
Jap S4^{ns}	1,000,000,000	0	0	0	0	110	88
Schu NS3	10,000,000	0	0	0	0	142	136

RESULTS

Persistence of different variants in vivo

Table 1 presents the results of the determination of the rate of multiplication of the variants in vivo. The data indicate that only the heterogeneous parent Jap culture and the variant, Jap S2, isolated from it, were capable of producing large numbers of organisms within the host. It is significant that the Jap S2 variant was the only type whose virulence was found to be similar to that of the heterogenous parent culture.[1] It is possible that a very small number of organisms might have been present in

the spleens of mice injected with other variants but were not detected by the methods employed. It may be noted that both ten and 14 days after vaccination the weight of the spleens from mice injected with the parent Jap or Jap S2 was greater than that of mice injected with the other variants.

When the more resistant white rat was used as the test animal in similar experiments, less significant results were obtained in regard to increase in the weight of spleens after vaccination. Nevertheless, 14 days after vaccination, the average spleen weight of rats inoculated with nonsmooth-reacting variants was far less than that of rats inoculated with smooth-reacting variants. As previously demonstrated for mice, organisms could be isolated for prolonged periods from the spleen of animals inoculated with the parent Jap culture or the Jap S2 variant, but not from animals inoculated with Jap S3, Jap S4^{ns}, or Schu NS3. It is noteworthy, however, that Jap S1, not isolated from mice, is capable of yielding detectable growth in rat spleens.

Immunogenic properties of the variants

In table 2, data are presented concerning the immunogenic potency of the several colony type variants when the mouse was used as the test animal. Fourteen days after vaccination, mice were tested for immunity with either 100 or 1000 subcutaneous LD_{50} of a highly virulent strain of *Bact. tularense.* Twenty animals were used at each test level. Fifteen days after administration of either LD_{50} test inoculum, 45% or more of the mice vaccinated with either the parent Jap culture or the Jap S2 variant remained alive. All mice vaccinated with the other variants died within nine days after inoculation with the highly virulent strain. These data were confirmed in a second trial with 280 mice. Similar results were also obtained when intraperitoneal, rather than subcutaneous, inoculation was used in an

TABLE 2.—*Immunogenic potency of colonial variants of* Bact. tularense *as determined by inoculation of vaccinated mice with highly virulent strain Schu S1.*

Strain or colony type variant used for preparation of living vaccine*	Number of LD_{50} of highly virulent strain Schu S1 administered subcutaneously to groups of 20 vaccinated mice	Number of animals found dead on the following days after administration of the LD_{50} test inoculum											Percentage survival
		1	2	3	4	5	6	7	8	9	10	11–15	
Jap (parent)	100	0	0	0	0	0	3	1	1	2	0	4	45
	1000	0	0	0	0	2	0	1	3	0	0	4	50
Jap S1	100	0	0	0	6	13	1						0
	1000	0	0	0	5	13	1	1					0
Jap S2	100	0	0	0	0	0	1	5	1	0	0	0	65
	1000	0	0	0	0	0	2	1	0	0	0	7	50
Jap S3	100	0	0	2	8	8	2						0
	1000	0	0	1	13	5	0	0	0	1			0
Jap S4^{ns}	100	0	0	7	11	2							0
	1000	0	0	1	18	1							0
Schu NS3	100	0	0	2	16	2							0
	1000	0	0	1	11	8							0
B. globigii	100	0	0	2	14	4							0
	1000	0	0	6	12	2							0
Control	100	0	0	1	17	2							0
	1000	0	0	9	10	1							0

* Mice were given a single subcutaneous inoculation of 10,000 viable cells and tested for immunity 14 days thereafter.

additional experiment employing 280 mice. The obvious differences in the protective ability of the colony type variants are not only detectable by differential survival but also by the time of survival between inoculation and death in the various groups. Inspection of data at the 100 LD_{50} level shows that no death occurred in mice vaccinated with the parent Jap culture or with the Jap S2 variant until six days after inoculation, whereas in the other groups, mice began to die two or three days after challenge. Inoculation of *B. globigii* 14 days prior to administration of the test inoculum failed to produce nonspecific protection.

In contrast to the results obtained with mice, vaccination of rats with any of the several variant types, smooth or nonsmooth-reacting, resulted in significant protection against subcutaneous inoculation with a highly virulent strain. Fifteen days after inoculation with the highly virulent strain, 60 to 100% of the rats vaccinated with either smooth- or nonsmooth-reacting variants of *Bact. tularense* survived at the 10,000 LD_{50} test level (table 3). Only ten animals were used in each group inoculated with 10,000 LD_{50}; slight differences in the percentage of survival, therefore, cannot be considered significant, and it must be concluded that all variants produced efficient protection. This general protective ability of the many different variants for rats, following subcutaneous challenge by a highly virulent strain, must be considered as specific since inoculation with *B. globigii* prior to administration of the test inoculum failed to produce any significant protection. All rats inoculated with *B. globigii* died within three days after challenge with 10,000 LD_{50}. Additional subcutaneous test data were obtained using a total of 160 rats, i.e., 20 animals in each group, of which 85 to 100% survived 100 LD_{50} if previously vaccinated with either the smooth- or nonsmooth-reacting variants. All nonvaccinated animals died within eight days after test inoculation.

TABLE 3.—*Immunogenic potency of colony type variants obtained from* Bact. tularense *strains Jap and Schu when the rat is used as the test animal.*

Strain or colony type variant used for preparation of living vaccine*	Percentage survival 14 days after inoculation with the highly virulent *Bact. tularense* variant Schu S1		
	Subcutaneous LD_{50} doses†	Intraperitoneal LD_{50} doses‡	
	10,000	10,000	1,000,000
Jap (parent)	100	80	85
Jap S1	60	60	40
Jap S2	80	90	80
Jap S3	80	75	35
Jap $S4^{ns}$	70	10	0
Schu NS3	60	30	15
B. globigii	0	10	5
Control (no vaccine)	0	5	0

* Rats were given a single subcutaneous vaccine inoculation of 10,000 viable cells and tested for immunity 14 days thereafter.
† 10 animals per test group.
‡ 20 animals per test group.

Although no significant differences in protective abilities between the various smooth- and nonsmooth-reacting variants were observed when vaccinated rats were subsequently challenged by the subcutaneous route, certain differences became apparent following intraperitoneal challenge. Results obtained when rats were vaccinated as previously, but tested for immunity by intraperitoneal inoculation with 10,000 or 1 million LD_{50}, are summarized in table 3. The percentage survival of the vaccinated rats inoculated with 1 million LD_{50} may be divided arbitrarily into four levels of protection: (a) efficient protection produced by either the parent Jap culture or the Jap S2 variant; (b) intermediate protection produced by smooth-reacting Jap variants, namely Jap S1 and Jap S3; (c) minimal protection produced by nonsmooth-reacting Schu type NS3; and (d) no protection by nonsmooth-reacting Jap $S4^{ns}$. Similar differences in protective abilities between smooth- and

nonsmooth-reacting variants are indicated in the results obtained at the 10,000 LD_{50} test level even though the differences between the smooth groups are somewhat less than observed in the larger inoculum. The negative results obtained by inoculation of *B. globigii* indicate that the low protective power of the nonsmooth-reacting Schu NS3 variant is specific.

DISCUSSION

The foregoing data have demonstrated that protection of vaccinated mice against virulent test inoculums administered by the subcutaneous or intraperitoneal route is produced only by the heterogeneous Jap culture or its component Jap S2 type. In contrast, all smooth- and nonsmooth-reacting variants tested protected rats against subsequent challenge with a virulent culture by the subcutaneous route, yet this uniform immunogenic behavior was not observed in rats when they were subsequently inoculated intraperitoneally with a virulent culture. Intraperitoneal inoculation of vaccinated rats revealed that protection was produced by all smooth-reacting types but that no significant protection was produced by nonsmooth-reacting types.

It is appropriate to suggest an explanation for the two major phenomena of intra- and interspecific host differences namely: (a) why can either smooth- or nonsmooth-reacting variants produce protection against subcutaneous inoculation of vaccinated rats whereas only certain smooth-reacting types produce protection in vaccinated mice and (b) why do both smooth- and nonsmooth-reacting types protect vaccinated rats against the subcutaneous inoculation, whereas only smooth-reacting types protect vaccinated rats against intraperitoneal inoculation?

The answer to the first question may be that a small amount of antigen capable of eliciting immunogenic response is present in all variants and may be sufficient to elevate the already existent high level of natural resistance of rats in such a manner that detectable protective effects against subcutaneous inoculation are produced. In contrast, the level of natural resistance in mice is extremely low and the relatively poor immunogenic potency of nonsmooth-reacting and certain smooth-reacting variants may not suffice to elevate resistance to appreciable levels. This would explain why smooth- and nonsmooth-reacting variants produced protection against subcutaneous inoculation in rats, but only one specific smooth type was capable of yielding comparable effects in mice.

The second question, regarding the differences in protection of vaccinated rats observed after subcutaneous versus intraperitoneal test inoculation of a highly virulent strain, may be answered by assuming that the decreased ability of nonsmooth-reacting variants to protect rats against intraperitoneal inoculation is due to by-passing, by intraperitoneal inoculation, a portion of the defense mechanism of rats which is operative when the subcutaneous route is used.

The ability of the several variants to protect the rat effectively, in contrast to the specific type requirement for mouse protection, is reminiscent of the differences in protection encountered when live and killed vaccines of *Bact. tularense* have been used in past experimental studies.[6,8,9] Killed vaccines are capable of protecting rats but fail to

8. Larson, C. L. 1945, Immunization of white rats against infections with *Pasteurella tularensis*. Pub. Health Rep. **60**: 725–734.
9. Ruchman, I. and Foshay, L. 1949, Immune response in mice after vaccination with *Bacterium tularense*. J. Immunol. **61**: 229–234.

protect mice, again indicating that only a weak antigenic stimulus is required to protect hosts of relatively high natural resistance against massive test doses. These observations are in good agreement with the considerations expressed above.

The analysis of immunogenic potencies of Jap culture variants confirms that cultures, formerly believed to be pure, actually may be heterogeneous not only in regard to colony type and virulence[1] but also in respect to immunogenic potency. The foregoing data show that effective protective capacity may be associated with only certain colonial types.

These results thus emphasize the importance of using selected clones in immunogenic studies of *Bact. tularense* or, for that matter, of any bacterial pathogen. Without recognition of these factors, certain cultures may be assumed to possess innate differences in immunogenic properties, whereas they may merely represent related heterogeneous populations containing different proportions of highly immunogenic and weakly immunogenic types. These factors can also account for such reports as that by Maisky,[10] who observed that the immunogenic properties of strains used for human vaccination varied and were difficult to maintain. Finally, the demonstration that certain variants, incapable of producing protective effects in the susceptible mouse, are capable of producing protection in the more resistant rat, serves as an example for the necessity of recognizing and controlling both inherent properties of the parasite and inherent levels of resistance of the host in immunological studies.

10. Maisky, I. N. 1949, Variants of the tularemia microbe due to external factors. Vestnik Akademii Meditsinskikh Nauk SSSR no. 5.

SUMMARY

Methods for the recognition of colony type variants of *Bacterium tularense* have made possible the isolation of variants from supposedly homogeneous stock cultures. Examination of these variants, as well as variants recovered from five-day static broth cultures, demonstrated that considerable differences in pathogenic and immunogenic properties may exist among variants and their heterogeneous parent culture. Only one of four variants, three smooth-reacting and one nonsmooth-reacting, isolated from a strain of relatively low virulence, was capable of immunizing the non-resistant mouse when administered as a live vaccine. Both smooth-reacting and nonsmooth-reacting variants were capable of immunizing the more resistant rat against subcutaneous inoculation with a highly virulent culture; however, only smooth-reacting variants were capable of immunizing the rat against intraperitoneal inoculation. Possible explanations for these observations have been discussed. The data presented demonstrate the importance of recognizing and controlling both inherent properties of the parasite and inherent levels of resistance of the host in immunological studies.

CAPSULE TYPES OF KLEBSIELLA

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Toennissen (1912, 1914, 1915, 1921a and b) was the first worker to draw attention to the serologic specificity of klebsiella capsules and to demonstrate the carbohydrate nature of the capsular antigens. Julianelle (1926a and b) clearly demonstrated the existence of three distinct capsule types of klebsiella, and Goslings and Snijders (1936) extended the number of capsular types to six. It was found by these workers that type C of Julianelle occurred typically in rhinoscleroma, while their new types, D, E, and F, were isolated from cases of ozaena. Up to this time the work on the capsular types of klebsiella, or Friedländer's bacillus, had been confined almost entirely to cultures from infections of the upper respiratory tract and from the sequelae of such infections.

As early as 1929 the senior author (Edwards, 1929) called attention to the similarity of the cultures of Friedländer's bacillus of that day to many cultures then classified as *Aerobacter aerogenes*. Not only were the two found to be so closely related that no basis for generic differentiation could be found, but many of the klebsiella and aerobacter cultures were biochemically and serologically identical. Parr (1939) and Borman, Stuart, and Wheeler (1944) also advocated that klebsiella and aerobacter strains should be included in one genus.

Recently, interest in the capsulated forms of the *Enterobacteriaceae* has been stimulated by their relative resistance to newer therapeutic agents. These forms have been found frequently in respiratory and urinary infections which did not yield readily to treatment with sulfonamides and antibiotics. Kauffmann (1949 and 1951) studied a number of cultures which possessed the characteristics generally attributed to Friedländer and aerobacter cultures. These nonmotile forms typically gave the IMVIC formula $--++$, and, in addition, they produced acid and gas from the majority of fermentable substances tested, including lactose, sucrose, adonitol, and inositol. Dulcitol usually was not fermented, hydrogen sulfide was not formed, but urea agar was slowly alkalinized. These characteristics served to differentiate the organisms, which Kauffmann characterized as the klebsiella group, from cultures of the escherichia group which typically possessed the IMVIC formula $++--$ and failed to ferment adonitol or inositol, or to alkalinize urea agar.

In a thorough study of 130 cultures derived from respiratory and urinary infections and which included classical strains as well as freshly isolated cultures, Kauffmann established 8 new capsule types and published the first antigenic classification of the klebsiella group based on the recognition of somatic (O) and capsular (K) antigens. Three O groups and 14 K antigens were established, and the organisms were divided into 18 types on the basis of these antigens. Since capsulated forms are not agglutinable in O serums and since the capsular antigens of klebsiella

Received for publication April 3, 1952.
The writers wish to express their gratitude to Dr. F. Kauffmann for his helpful advice and for furnishing numerous cultures and samples of serum. The technical assistance of Lt. G. M. Moore (USNR) is gratefully acknowledged.

strains are heat stable, it was necessary to obtain acapsular forms in order to determine the O antigens of the cultures, or alternatively, to prepare serums from capsulated forms for testing with acapsular strains representative of known O groups. The determination of O antigens of klebsiella often is a difficult and time-consuming process, and the methods used in the isolation of acapsular forms may lead to the production of rough variants of changed antigenic constitution. Hence, the practical serologic identification of klebsiella cultures rests upon the recognition of capsular antigens.

The present study was undertaken since, in the course of reference diagnostic work, repeated requests for the typing of klebsiella cultures were received. Serums for the 14 capsule types delineated by Kauffmann were prepared. Surprisingly, only a small percentage of the cultures other than those of capsule types 1 and 2 (Friedländer A and B) could be typed with these serums. Therefore, it was necessary to prepare serums for a number of additional types. During the course of the work five cultures which belonged to the same capsular type and serum prepared from one of them were received from Dr. William Ferguson, who had isolated the forms from an outbreak of infant diarrhea (Worfel and Ferguson, 1951). The results obtained by Worfel and Ferguson were confirmed, and it was found that the capsular antigens of this type were unrelated to those previously described. The organisms were designated as capsule type 15.

While the work was in progress, Brooke (1951), working in Kauffmann's laboratory, recognized 27 additional capsule types of klebsiella cultures. Since cultures were being reported by the writers under the designation of capsule types 15 to 25 inclusive, and since it was not known whether the Brooke types were identical with those established in this laboratory, Dr. Brooke considerately labeled his types as provisional types Br. 15 to Br. 41 inclusive. Through the courtesy of Dr. Kauffmann, serums and cultures representative of the Brook types were made available. It was found that only three of the Brooke types were identical with the types recognized by the writers.

Sources of cultures

Cultures of types 1 to 14 inclusive and the 27 provisional types of Brooke (1951) were received from Dr. F. Kauffmann. These were as shown in table 1. In addition to the 41 type cultures, 215 other cultures were examined. The sources of these were as shown in table 2. These cultures did not constitute a selected material but represented the

TABLE 1.—*Source of cultures of types 1 to 14 and the 27 provisional types of Brooke (1951).*

Type	Culture		Source
1	A5054	*K. pneumoniae*	From the National Collection of Type Cultures, London
2	B5055	*K. pneumoniae*	
3	C5056	*K. pneumoniae*	
4	D5050	*K. ozaenae*	
5	E5051	*K. ozaenae*	
6	F5052	*K. ozaenae*	
7	4140	*Bact. lactis aerogenes*	
8	1015		
9	56		
10	919	Klebsiella cultures isolated from urine (Kauffmann, 1949)	
11	390		
12	313		
13	1470		
14	1193		
16	7666	Provisional Brooke type 20	Pus, peritoneum
23	5473	" " " 37	Urine
24	9291	" " " 30	Urine
26	5884	" " " 15	Expectorate
27	6613	" " " 16	Urine
28	5758	" " " 17	Urine
29	5727 Y	" " " 18	Expectorate
30	7824	" " " 19	Urine
31	6258	" " " 21	Pus, wound
32	6837	" " " 22	Expectorate
33	6168	" " " 23	Urine
34	7522	" " " 24	Urine
35	7444	" " " 25	Gall Bladder
36	8306	" " " 26	Urine
37	8238	" " " 27	Urine
38	8414	" " " 28	Urine
39	7749	" " " 29	Urine
40	8588	" " " 31	Urine
41	6177	" " " 32	Throat swab
42	1702	" " " 33	Uterine secretion
43	2482	" " " 34	Urine
44	7730	" " " 35	Pus, nose
45	8464	" " " 36	Urine
46	5281	" " " 38	Expectorate
47	9682	" " " 39	Pus, wound
48	1196	" " " 40	Urine
49	6115	" " " 41	Urine

TABLE 2.—*Additional cultures examined other than the 41 type cultures (table 1).*

Source	Number of cultures examined
Sputum and bronchial exudate	68
Lungs and pleura	19
Urine and genito-urinary tract	60
Stools and intestinal content	19
Pus and abscesses	14
Blood	14
Bones and joints	4
Ozaena	9
Miscellaneous sources	8
	215

klebsiella cultures received in the laboratory during the course of the investigation.

METHODS

Inasmuch as close attention to detail is absolutely necessary to successful typing of klebsiella cultures it seems desirable to describe briefly the methods used in the work.

Biochemical tests.—The cultures studied were subjected to the following tests: methyl red; Voges-Proskauer; utilization of citrate, urea, and D-tartrate; reduction of nitrate; production of hydrogen sulfide and indol; liquefaction of gelatin; and fermentation of glucose, lactose, sucrose, dulcitol, inositol, adonitol and salicin. Simmons' citrate agar was used to test utilization of citrate, and O'Meara's method was used in the Voges-Proskauer test. Christensen's urea agar was employed in tests for utilization of urea and Jordan's tartrate agar in tests for utilization of D-tartrate. Hydrogen sulfide production was judged by appearance in triple sugar iron agar and Kovac's test was used to detect indol. Fermentable substances were incubated for 30 days before they were recorded as negative.

Determination of capsules.—The most satisfactory method found to determine the number and size of capsules present in a culture was moist India ink mounts. A droplet of a dilute suspension of cells was placed on a slide and a very small droplet of Pelican India ink placed adjacent to it. The two were joined with a loop and a cover slip immediately placed thereon without mixing. The cell suspension and ink flowed together so that some portion of the preparation possessed the opacity best suited for the determination of capsules.

Preparation of capsular antiserums.—Antiserums were prepared by injecting rabbits with cultures which possessed well-developed capsules. Two types of material were injected. The first consisted of cultures grown for 4 to 6 hours in infusion broth containing 0.2% glucose and killed by the addition of 0.5% formalin, as suggested by Kauffmann (1949). Other rabbits were injected with organisms which were grown on infusion agar plates, from which they were scraped and suspended directly in absolute alcohol. After standing overnight at room temperature the organisms were sedimented by centrifugation and the fluid decanted and replaced with an equal volume of alcohol. After again standing overnight the organisms were again centrifuged and the alcohol decanted. The sedimented bacteria were dried in vacuo, ground to a fine powder and stored in stoppered tubes at room temperature. The animals were given 5 injections of 0.3, 0.5, 1.0, 2.0, and 2.0 ml of broth culture at intervals of 4 days. They were then trial bled, and if the titer was satisfactory the animals were bled out on the 6th day after the last injection. If an acceptable titer was not obtained after 5 injections, two additional doses of 2.0 ml each were given. If the titer was still unsatisfactory the rabbits were discarded.

In injecting the alcohol-treated bacteria the cells were suspended in physiological saline immediately before injection and were administered in the volumes mentioned above. The first injection was of approximately the same density as the broth cultures, but as the amounts injected were increased, the density of the suspensions also was increased, until very heavy suspensions were reached. On the whole, there was little difference in the strength of the serums produced with the two types of antigen. Inasmuch as the formalinized broth antigens are much more easily prepared, they are preferable to alcohol-treated antigens.

Slide agglutination tests.—Dense suspensions of well-capsulated cells in phenolized saline were tested with undiluted individual serums in the usual manner. When the suspensions contained few or no acapsular forms, O agglutination was completely masked and capsular agglutination was immediately apparent. If the suspensions were not well capsulated, O agglutination occurred in many of the serums since the O antigens of many of the cultures are related and the O titers of the serums greatly exceeded the capsular titers.

Tube agglutination tests.—Cultures incubated for 4 to 6 hours in infusion broth which contained 0.2% glucose were preserved by the addition of 0.5% formalin and used as antigens in tube agglutination tests. Each type culture was tested with each serum in serial dilutions. The lowest dilution employed was 1:4. Tests were incubated overnight at 48 C and read. As in the case of slide agglutinations, it was necessary to use only antigens in which practically all the cells were capsulated in order to suppress O agglutination. As is well known, capsular agglutination is evidenced by the formation of a firm disc precipitate which is dispersed only with difficulty. On the contrary,

O agglutination is evidenced by a granular precipitate which forms much more slowly. In spite of the diverse appearance of the two reactions, it is extremely difficult to establish the limits of capsular agglutination in a suspension which contains both capsulated and noncapsulated elements. Therefore, it is necessary that only uniformly capsulated suspensions be employed as antigens.

Agglutinin absorption tests.—Absorption tests were performed in each instance in which capsular relationships were apparent in slide or tube agglutination tests. The growth from one agar plate was taken up in a small amount of phenolized saline so that the total volume of the suspension was approximately 0.8 ml. This suspension was mixed with 0.8 ml of the serum to be absorbed and incubated at 48 C for one hour. The suspension was centrifuged and the supernatant serum made up in serial dilutions and tested with the homologous and absorbing suspensions. As a rule, agglutinins for the absorbing culture were removed by this process, but occasionally the absorption was incomplete and it was necessary to absorb the serum twice in order to remove all heterologous capsular agglutinins.

Capsular reactions.—For the capsular or Quellung reactions dilute suspensions of the organisms were prepared in phenolized saline. Methylene blue was not added to the suspensions, as often is done, since the presence of the dye did not aid in the recognition of the reactions. Each type strain was tested with each of the serums by mixing one loopful of suspension and one loopful of undiluted serum on a slide. Cover slips were added and the mixtures examined microscopically under an oil immersion lens. In those instances in which a Quellung reaction was noted the suspensions were tested with serial dilutions of serum to determine the titer of the reaction.

In the performance of capsular reactions it is important that the suspensions contain only a relatively small number of cells; 4 to 8 cells per oil immersion field is a satisfactory number. If the organisms are too numerous, large agglutinated masses may result so that it is difficult to distinguish capsular reactions. In addition, it is advisable to use only freshly prepared suspensions since suspensions which have been made up for some time are likely to contain soluble specific substance which unites with the antibody to form precipitate at the expense of the Quellung reaction.

RESULTS

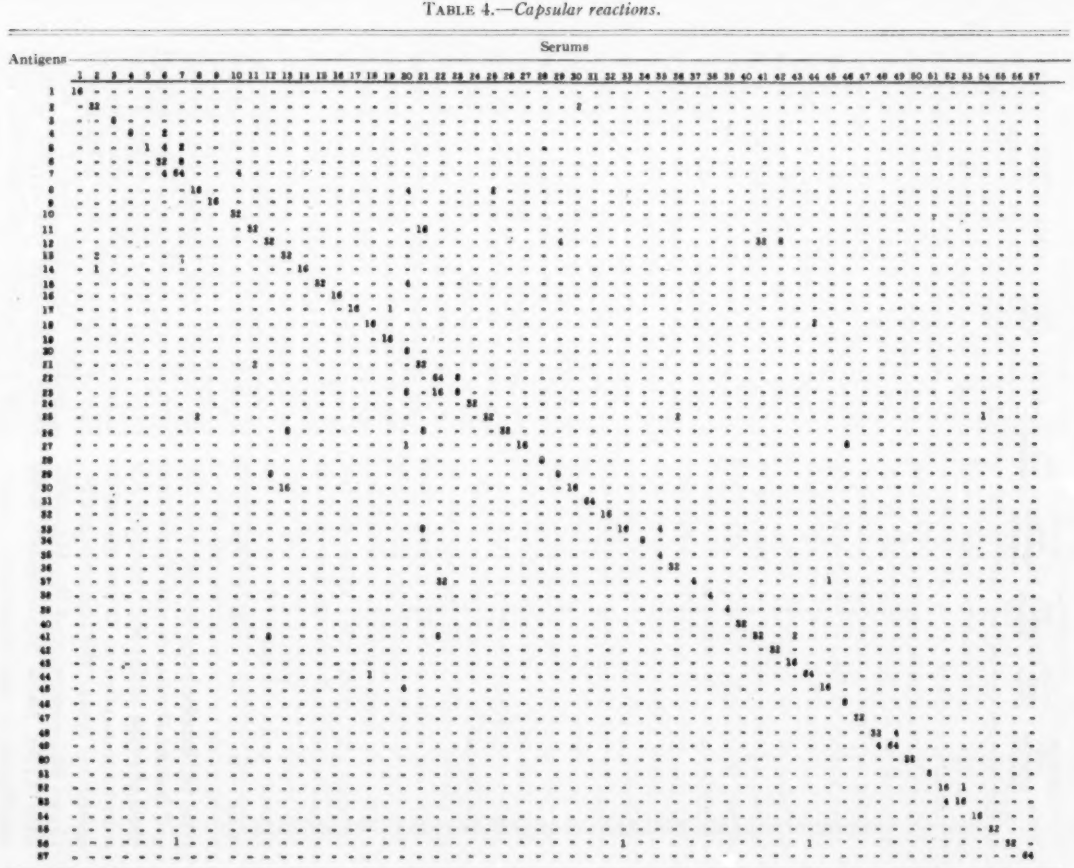
Biochemical reactions.—From the literature it can be concluded that the typical biochemical reactions of klebsiella strains are as follows: methyl red, hydrogen sulfide, and indol tests are negative and dulcitol is not fermented. Voges-Proskauer, citrate, urea, tartrate, and nitrite tests are positive; and glucose, lactose, sucrose, inositol, adonitol, and salicin are fermented with gas production. Of the 256 cultures examined, 151 differed from the above pattern in one or more respects. Aside from the unusual characteristics of rhinoscleroma strains and the fermentation of dulcitol by type 10 (Kauffmann, 1949), the aberrations in biochemical characteristics were not related to type. Therefore, it does not seem necessary to list the reactions of the cultures type by type. Suffice to say that 31 cultures were methyl red positive, 25 were Voges-Proskauer negative, 11 produced indol, 3 produced hydrogen sulfide, 28 failed to alkalinize urea agar, 10 failed to utilize tartrate, 1 failed to reduce nitrate, 1 liquefied gelatin, 9 were anaerogenic, 30 failed to grow on Simmons' citrate agar, 4 did not ferment lactose, 6 did not ferment sucrose, 6 did not ferment inositol, 20 did not ferment adonitol, 5 did not ferment salicin, and 59 fermented dulcitol.

Serologic reactions.—The results of the tube agglutination tests and capsular reactions, respectively, are set forth in tables 3 and 4. The results of slide agglutination tests are not tabulated since they parallel closely the tube agglutination tests and the capsular reactions. It is evident that, as in the pneumococcus group, intertype relationships exist, but each of these related types was shown to be antigenically distinct by reciprocal absorption tests. Types 1 to 14, inclusive, are the types established by Kauffmann (1949), type 15 is the type described by Worfel and Ferguson (1951), and types 16 to 25, inclusive, are types established in this laboratory. Three of the provisional Brooke types were found to be identical with types established by the writers. Brooke type 20 was identical with

TABLE 3.—*Tube agglutination.*

| Antigens* | Serums | | |

TABLE 4.—*Capsular reactions.*



See footnotes of table 3.

TABLE 5.—*Sources of klebsiella types.*

Types	Sputum and bronchial exudate	Lungs and pleura	Urine and genito-urinary tract	Stools and intestinal materials	Pus and abscesses	Blood	Bones and joints	Ozaena	Miscellaneous	Total
1	15	3	3	1	4	1	3		1	31
2	18	2	5		2	4	1			32
3	1				1	1			1	4
4	10	1	1					7		19
5	3							2		5
6	2							2		4
7	1		5	2	1				1	10
8			2							2
9		2	5							7
10			4			1				5
11	1		3							4
12	2		2							4
13	1		2							3
14	4	1	3							8
15	1		3	5		1				10
16			2		1	2				5
17	2		6	3	1					12
18	2									2
19	4		4			1				9
20		1	2	3	2	2				10
21		1	4	1	1				1	8
22		2	1						1	4
23		1	1							2
24			1	1						2
25			2							2
26	1									1
27			2							2
28			2							2
29	1									1
30			1							1
31		1	1		1					3
32	1									1
33			1							1
34			1							1
35	1	1	1		1			1	2	7
36			1							1
37			1							1
38		1	1							2
39			1							1
40			1							1
41	1		1							2
42			1							1
43			1							1
44			1		1					2
45			1							1
46	1									1
47					1					1
48			1							1
49			1							1
50									1	1
51	1				1					2
52		1	1			1				3
53				1					1	2
54		1	1	1						3
55	1			1						2
56			1							1
57	1									1
Total	76	19	85	19	18	14	4	12	9	256

type 16, Brooke type 30 with type 24, and Brooke 37 with type 23. The 27 provisional types of Brooke, therefore, are reduced in number to 24 and are represented by types 26 (=provisional Br. 15) to 49 (=provisional Br. 41), inclusive. Types 50 to 57, inclusive, were established in this laboratory.

The sources of the cultures of the various types are given in table 5. Among the cultures from miscellaneous sources, one culture of type 3 was isolated from rhinoscleroma, one culture of type 21 was found in sinusitis, and one culture of type 35 was isolated from an infected gall bladder. One culture each of types 7, 15 and 22 was isolated from water. One culture of type 35 was isolated from a bottle of tonic. One culture of type 50 was found in the liver of a turkey. Although type 1 is rarely found in animals, one culture of that type was isolated from the spleen of a monkey.

The salient facts regarding the antigenic relationships of the types are set forth in the tables so that it seems unnecessary to discuss them at length. It is evident that some of the types are related to a sufficient extent that diluted

or absorbed serums must be employed to distinguish them. It usually is possible to distinguish related types one from another by determining the titers of the capsular reactions of a given culture in the serums with which it reacts. However, absorbed serums are more satisfactory and can be prepared without too great difficulty. A schema for the absorption of serums for specific fractions was outlined by Brooke (1951). Intertype relationships are most troublesome in case of types 4, 5, 6, and 7, since these are the only types which have close relationships and which occur frequently. In absorbing the nonspecific antibodies from the serums it usually is not necessary to use such large doses as when reciprocal absorptions are done to determine the identity or nonidentity of two related types. In practice it has been found possible to add very dense suspensions of the organisms, prepared by suspending the growth from agar plates in a minimal amount of saline, to undiluted serum drop by drop until agglutination is no longer apparent. Agglutination of capsulated organisms by undiluted serum takes place so rapidly that it is possible to determine roughly the point of saturation if the organisms are dropped slowly into the serum which should be constantly agitated. The organisms to be used in any absorption are dictated by the results of cross agglutination and cross Quellung tests. After absorption the specific serums may be used in slide agglutination and Quellung tests for exact type determination.

The relation of slime to capsular substance.—Kauffmann (1949) very clearly stated the various forms in which klebsiella may occur. The antigens of smooth forms were expressed as follows:

1. MKO form—mucoid, capsulated, with O antigen
2. KO form—nonmucoid, capsulated with O antigen
3. MO form—mucoid, acapsular with O antigen
4. O form—nonmucoid, acapsular with O antigen

All of the forms mentioned above have been encountered during the course of the work. Inasmuch as the O and MO forms are acapsular, their typing is dependent upon the isolation of capsulated forms by growth on an appropriate medium and by colony selection. The medium of Worfel and Ferguson (1951) solidified by the addition of 2% agar has proved most helpful in obtaining regularly capsulated forms from cultures which on initial observation exhibited only a very few capsules. It is obvious that acapsular forms cannot be typed by Quellung reactions since they are surrounded by no capsular substance with which serum can react. Occasionally such cultures may be quite mucoid and slimy. One such culture is strain 1193 of Kauffmann (1949), which is the type strain of capsule type 14. On plating, this culture breaks up into opaque colonies which are nonslimy but capsulated (KO form) and translucent colonies which are slimy but acapsular (MO form). In spite of the fact that the MO form possesses no visible capsules, it is quite mucoid and the colonies draw out into long threads when touched with a needle. The question of the relation of the slime of the MO form to the capsule of the KO from naturally arises. It is believed that the following experiment throws some light on this question.

The KO and MO forms of culture 1193 were selected carefully and inoculated into infusion broth which contained 0.2 glucose. After overnight incubation, examination of the cultures revealed regularly capsulated forms in the KO culture, while no capsules were found in the culture of the MO form, although the broth was visibly slimy. The cultures were centrifuged to clarity

TABLE 6.—*Ring tests with supernatant fluid of broth cultures.*

Antigens	Serums	Antigen dilutions									
		1	2	4	8	16	32	64	128	256	512
1193, mucoid, acapsular (MO) form	F26	–	–	–	–	–	–	–	–	–	–
	F36	–	–	–	–	–	–	–	–	–	–
	F46	+	+	+	+	+	+	±	±	–	–
	F47	+	+	+	+	+	+	±	–	–	–
1193, nonmucoid, capsular (KO) form	F26	–	–	–	–	–	–	–	–	–	–
	F36	–	–	–	–	–	–	–	–	–	–
	F46	+	+	+	+	+	+	+	–	–	–
	F47	+	+	+	+	+	+	+	–	–	–

0.2 ml serum used in all tests. Incubation—1 hour, room temperature.

TABLE 7.—*Precipitin tests with supernatant fluid of broth cultures.*

Antigens	Serums	Antigen dilutions									
		1	2	4	8	16	32	64	128	256	512
1193, mucoid, acapsular (MO) form	F26	–	–	–	–	–	–	–	–	–	–
	F36	–	–	–	–	–	–	–	–	–	–
	F46	×	×	×	×	×	×	+	+	–	–
	F47	×	×	×	×	×	×	×	+	–	–
1193, nonmucoid, capsular (KO) form	F26	–	–	–	–	–	–	–	–	–	–
	F36	–	–	–	–	–	–	–	–	–	–
	F46	×	×	×	×	×	+	–	–	–	–
	F47	×	×	×	×	×	–	–	–	–	–

0.2 ml serum used in all tests. Incubation—overnight, 50 C.
× =disc precipitate, + =granular precipitate.

TABLE 8.—*Agglutination tests with formalinized broth cultures.*

Antigens	Serums	Serum dilutions							
		4	8	16	32	64	128	256	512
1193, mucoid, acapsular (M) form	F26	+	+	+	+	+	+	+	+
	F36	+	+	+	+	+	+	+	+
	F46	+	+	+	+	+	+	+	+
	F47	+	+	+	+	+	+	+	+
1193, nonmucoid, capsular (KO) form	F26	–	–	–	–	–	–	–	–
	F36	–	–	–	–	–	–	–	–
	F46	×	×	×	×	×	×	–	–
	F47	×	×	×	–	–	–	–	–

+ =0 agglutination.
× =capsular agglutination.

and the clear supernatant broths were used as antigens in precipitin tests. Undiluted serum was added to each tube in amount of 0.2 ml. On the serum was layered 0.2 ml of serial dilutions of the two centrifuged broth cultures. Four serums were available for use in the tests. Two of these serums, F26 and F36, were derived from rabbits injected with the MO form of 1193, while the other two, F46 and F47, were from rabbits injected with the KO form of the culture. The tests were incubated at room temperature for one hour and the ring tests read. The tubes were then shaken, incubated in the water bath at 50 C overnight and again observed. The results of these tests are given in tables 6 and 7.

At the same time, four-hour infusion broth cultures of the KO and MO forms were formalinized and used as antigens in agglutination tests with the four serums mentioned above. The results of the agglutination tests are given in table 8.

From the tables it may be seen that the supernatant of the MO form gave a positive ring test in a higher antigen dilution (1:128) than did the KO form (1:64). Likewise, upon incubation the MO form was precipitated in higher dilution (1:128) than was the KO form (1:32). In both antigens the precipitates

in the lower dilutions consisted of the discs characteristic of the polysaccharide antigens usually present in capsular material. It should be noted also that the serums of the two rabbits injected with the MO form failed to precipitate the antigens.

The agglutination tests confirmed the observations made in the precipitin tests in that the serum of rabbits injected with the MO form failed to agglutinate capsulated bacteria. On the contrary, the acapsular MO form was completely agglutinated in the highest dilution tested by both KO and MO serums. In contrast to the discs observed in the KO antigens, the MO form was agglutinated in the granular manner characteristic of O antigens. This substantiates the observation of Kauffmann (1949) that MO forms, having no capsules or envelope antigens, are susceptible to the action of O agglutinins.

DISCUSSION

The results of the biochemical tests indicate that while it is possible to establish a pattern of biochemical behavior for the klebsiella group, frequent deviation from this pattern must be expected. Of 256 cultures included in the study, 151 differed from the pattern in one or more characteristics. Kauffmann (1949) found that rhinoscleroma strains were biochemically distinct members of the group and that capsular type 10 fermented dulcitol. Thus, while these deviations from the pattern were to be expected, they accounted for only a small percentage of the aberrant results observed. Further, several types based on type strains which did not conform to the regular pattern later were found to include biochemically typical cultures. Instances of this are to be found in type 1, in which the type strain was MR positive, VP negative, and anaerogenic, and in type 4, in which the type strain possessed the same properties as the type 1 strain and, in addition, failed to reduce nitrates.

Twenty-one of the 24 types established by Brooke were founded on cultures which did not follow the established pattern; a number of these cultures were indol positive and two approached the IMVIC pattern of *Escherichia coli*. Nevertheless, it was found that types based on indol positive cultures may encompass cultures which are biochemically typical. This situation should not be unexpected since Kauffmann demonstrated that certain capsular (A) antigens of escherichia cultures were closely related to the capsules of klebsiella types. Similar relationships have been noted in the course of this work. Encapsulated cultures of *Esch. coli* were encountered which were agglutinated and Quelled strongly by the serums of klebsiella types 16 and 21.

While it is well known that the family of enteric bacteria is composed of a series of interrelated types which are not readily divisible into exact groups, the value of biochemical tests in the examination of klebsiella cultures should not be minimized. Although many strains may vary from the norm in one or more characteristics, biochemical reactions are a helpful and necessary adjunct to their typing.

The type distribution of klebsiella cultures isolated in this country is worthy of note. Types 1 to 6 (A to F) have been known for many years to be associated with infections of the upper respiratory tract. If the type strains, which were of European origin, and 5 type D strains, received by Dr. M. Frobisher from Jugoslavia and handed to the writers, are excluded from the total, 84 cultures of types 1 to 6, inclusive, were recognized. Of these, types 1 and 2 greatly outnumbered the others, although type 4 occurred frequently. The majority of the cultures of these types was isolated from upper respiratory infections but they were found also in genito-urinary infections, in septi-

cemias and in various localized purulent processes.

The 8 types established by Kauffmann (types 7 to 14, inclusive) were represented only by 35 cultures isolated in the United States. The marked predominance of types 8, 9, and 10, noted both by Brooke and Kauffmann, was not evident in this study. Kauffmann (1949) assigned 93% of urinary strains to these types, while Brooke (1951) placed 63% of the urinary strains which he studied in types 8, 9, and 10. In the present study these types constituted 16% of 85 cultures isolated from urine and genito-urinary tract. This wide difference in incidence confirms Kauffmann's statement that type distribution would be subject to variations due to difference in time and location of studies.

The 24 provisional types of Brooke which proved to be distinct from types established in this study (types 26 to 49, inclusive) accounted for but 13 cultures, or 6% of the strains isolated in the United States. In Brooke's study they represented 16% of 310 cultures. Only one of the types, type 35 (provisional Br. 25), was found with any degree of frequency in this country.

The 19 types based on cultures isolated in the United States (types 15 to 25, inclusive, and 50 to 57, inclusive) were represented by 81 cultures, or 38% of the strains isolated in this country. From the foregoing, it is obvious that, aside from the widely known types A, B, C, D, E, and F, the incidence of the various klebsiella types in Denmark and in the United States is quite different.

Some remarks regarding capsules and capsular (Quellung) reactions seem justified. It is well known that capsulation in the klebsiella group is subject to marked variation and that many strains quickly lose their capsules on artificial cultivation. Upon plating klebsiella cultures, opaque and translucent, and mucoid and nonmucoid forms often are apparent. A priori, one would judge that either the opaque colonies or the translucent colonies regularly would represent the capsulated form. In the experience of the writers such is not the case. In some cultures translucent, mucoid colonies are surrounded by large amounts of capsular substance, while opaque, nonmucoid colonies are devoid, or almost devoid, of capsules. In other cultures exactly opposite conditions occur and the opaque, nonmucoid colonies are capsulated. It is advisable and, in fact, necessary to examine each individual culture in order to determine which colony type is capsulated. The differences in colony appearance often are exaggerated when the culture are plated on the medium of Worfel and Ferguson. A case in point is the type strain of type 5 (E5051), which, in Kauffmann's experience, produced no capsules, yet which through colony selection has yielded a variant surrounded by the largest accumulation of capsular substance of any strain examined. The peculiar behavior of the type strain of type 14, mentioned above, was brought to the attention of the writers by Dr. Kauffmann.

Undoubtedly, objections will be raised to the method used here for the determination of capsules. It will be pointed out that in moist India ink preparations it is not possible to distinguish between a slime layer accumulated around the organism and a definite capsule with a limiting membrane. It is true that such a distinction cannot be made, but from a practical standpoint, moist India ink preparations with living cells are by far the simplest and most accurate method yet devised for determining the percentage of cells in a given culture which are surrounded by a capsule or slime layer which will inhibit O agglutination. From observation of such preparations it usually is possible to predict whether a culture will prove to be O agglutinable or whether it may be typed by capsular

agglutination tests. Very often it is possible to observe the gradual dissolution of the slime layer under the microscope. In portions of the preparation in which streaming occurs one will observe cells which are held stationary. Study of such cells reveals small bits of the capsular material streaming out in threadlike projections from the capsular mass. These projections are agitated by the moving India ink particles, and their appearance somewhat resembles that of spirochaetes in a dark field preparation. These particles of capsule or slime become detached from the mass, float about free in the preparation, and gradually disintegrate. It seems very likely that in some cultures it is by such a process that precipitable substances appear in the culture fluid. If Quellung tests are done with a suspension in which this process of dissolution is taking place, precipitation occurs in the suspending medium, sometimes to such an extent that the capsular reaction fails or is obscured. Failure of Quellung reactions due to antigen excess is well known.

From these observations and from the experiments with slimy, noncapsulated cultures, it is evident that both slime and capsular substance react similarly in serologic tests. Also, from direct microscopic examination of India ink preparations of living cells and from corresponding Quellung reactions, it is evident that the entire mass surrounding the cell, whether it be true capsule or slime layer, is involved in Quellung tests. The writers are in agreement with Kleineberger-Nobel (1948), who stated that in this family of bacteria no swelling of the capsule occurs when the organisms are placed in contact with specific serum. A precipitin reaction occurs at the surface of the capsule or slime layer, causing the margin of the mass to become highly refractile and to simulate the appearance of a cell wall. In hundreds of observations of cells in parallel India ink and serum preparations, swelling of the mass surrounding the cells has never been observed.

The reason for the accumulation of slime around the cells of some cultures and for its immediate dissipation into the medium in other cultures is not clear. The fact that variants in which both conditions occur can be found on the same plate of a pure culture indicates that it is a function of the cell and not of the medium. Both variants tend to breed true, although the slimy, acapsular form is more stable. It is much easier to isolate slimy, acapsular variants from a capsulated culture than to isolate capsulated forms from a slimy, acapsular culture. As noted above, in the writers' experience, slimy, acapsular forms have failed to elicit capsular antibodies when injected into rabbits. Further, the mucous antibodies mentioned by Kauffmann were not demonstrated in serum produced with slimy forms of types 5 and 14. It is rather difficult to reconcile these results with the observation of Brooke that satisfactory capsular serum could be produced by the injection of the supernatant fluid of centrifuged broth cultures of the capsulated form. In retrospect, it was found that cultures which produced distinct but relatively small capsules and which were not mucoid provided the most satisfactory antigens for serum production. Little difficulty was encountered in producing satisfactory serums from such cultures, whereas heavily capsulated, mucoid forms caused much difficulty in serum production. The only type 5 serum of satisfactory titer in the writers' possession was one received through the courtesy of Dr. Morris Shaffer. This serum, unlike that described by Brooke, retained a specific agglutinin and Quellung titer after absorption by other types.

In the sixth edition of Bergey's Manual (1948) the genus *Klebsiella*

contains only nonmotile forms, while the genus *Aerobacter* is made up of both motile and nonmotile forms. There is no further difference in the characterization of the two genera, and it is evident that the two should be combined, as recommended by Edwards (1929). No basis for the separation of the two, either upon type of disease produced or upon biological characteristics, is known. It is true that our knowledge of the O antigens of the genus is slight, being confined to the observations of Kauffmann (1949), and nothing is known of the H antigens of the motile cultures. Although the work described here is concerned only with nonmotile forms, motile forms which possess the same biochemical characteristics occur. Occasionally, capsulated cells are present in the motile cultures, and it is possible to isolate from them variants which have well-defined capsules and which can be typed by capsular agglutination and by Quellung reactions. It would appear, therefore, that the klebsiella group should include both motile and nonmotile forms and should be classified antigenitally through determination of O, K, and H antigens, just as the *Esch. coli* group is classified. Up to the present, all the emphasis has been placed on capsular antigens. This situation is comparable to a classification of *Esch. coli* which was based only upon the recognition of A antigens. The study of the group should continue until a satisfactory classification is achieved, even though practical identification rests upon recognition of capsule types.

SUMMARY

A biochemical and serological study was made of 256 nonmotile, encapsulated cultures classified as klebsiella. Of these, 41 were type cultures of European origin and 215 were cultures received for diagnosis. Although biochemical methods were of great assistance in the classification of the cultures, variation from the predominant biochemical type frequently was observed.

Through agglutination, absorption, and Quellung tests the organisms were divided into 57 capsular types. Of the 57 capsular types, 39 were previously described while 18 were encountered for the first time in this study. There was a marked difference in incidence of capsular types in the United States and in Denmark.

The slime of mucoid, acapsular cultures was closely related serologically to the capsular substance of encapsulated, nonmucoid cultures. No swelling of capsules was observed in Quellung reactions.

After the above manuscript was written there came to the attention of the writers a paper by Duguid (1951, J. Path. & Bact. **43**: 673) in which it was concluded that moist India ink mounts were by far the most satisfactory method for the determination of capsules. Further, it was emphasized that no swelling of the capsular material occurred in Quellung tests with pneumococci. The observations made in the present work confirm the conclusions of Duguid.

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